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(54) Title: PHEROMONE COMPOSITIONS AND METHODS OF USE IN CONTROLLING FUNGAL DISEASES IN PLANTS

(57) Abstract

Disclosed are pheromone compositions comprising fungal mating factors, methods for making and using native and recombinant pheromone compositions and derivatives thereof in interfering with fungal pathogenesis, and methods for making and using these compositions for preventing fungal infection and disease in plants.

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PHEROMONE COMPOSITIONS AND METHODS OF USE IN CONTROLLING FUNGAL DISEASES IN PLANTS

1. BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of U. S. Provisional Patent Application Serial Number 60/019,598 filed June 17, 1996, the entire contents of which is specifically incorporated herein by reference. The United States government has rights in the present invention pursuant to Grant MCB-9205818 from the National Science Foundation, Grant 94-37303-0572 from the United States Department of Agriculture, and Grant GM47977 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates to the field of molecular biology. More specifically, it concerns pheromone compositions comprising fungal mating factors, methods for making and using native and recombinant pheromone compositions and derivatives thereof in interfering with fungal pathogenesis, and methods for making and using these compositions for preventing fungal infection and disease in plants.

1.2 DESCRIPTION OF THE RELATED ART

1.2.1 PLANT FUNGAL PATHOGENS

Magnaporthe grisea is an ascomycete with two mating types, termed *MAT1-1* and *MAT1-2*. It is a pathogen of a wide variety of grasses and is best known because of its potentially devastating impact on rice production. The costs of disease control by fungicides and the difficulty in breeding durable and effective resistance to this disease have led to intense interest in understanding the biology of the pathogen. Fortunately, the sexual cycle can be exploited in the laboratory and many of the research techniques developed from the more tractable filamentous ascomycetes such as *Neurospora crassa* and *Aspergillus nidulans* have been successfully adapted for use with *M. grisea* (Valent and Chumley, 1991).

M. grisea initially attaches to the surface of the host by a glue, termed spore tip mucilage, that is released upon hydration of the conidium (Hamer *et al.*, 1988). Following germination, the fungus responds to contact with a potential host surface by

producing an appressorium. The appressorium of *M. grisea* develops by swelling of the hyphal tip to form a dome-shaped cell containing several new cell wall layers (Howard, 1994). One of these wall layers is composed of melanin that acts as a barrier to solute movement. An increase in solute concentration within the cell forces water to enter, thus increasing the turgor pressure of the cell tremendously (Howard *et al.*, 1991). This physical pressure aids in the penetration of the host cell wall by an infection hypha (Howard *et al.*, 1991).

A diverse group of plant pathogenic fungi form appressoria (Emmett and Parbery, 1975). Physical and chemical signals that trigger appressorium formation can include the height of the ridges between stomatal guard cells of the host plant (Wynn, 1976; Hoch *et al.*, 1987), the presence of host-specific fatty alcohols (Kolattukudy *et al.*, 1995), host surface properties (Lee and Dean, 1993) and other environmental cues (Jelitto *et al.*, 1994). The environmental signals inducing development are unique to the individual organisms, *i.e.*, ridges induce appressorium formation in *Uromyces appendiculatus* (Wynn, 1976; Hoch *et al.*, 1987), but not *M. grisea* (Lee and Dean, 1994). In *M. grisea*, addition of cAMP activates development on surfaces that lack specific inductive cues (Lee and Dean, 1993), and a cAMP-dependent protein kinase mutant is defective in appressorium development (Mitchell and Dan, 1995). Protein kinase inhibitors can block development in Colletotrichum (Kolattukudy *et al.*, 1995). cAMP and phophodiesterase inhibitors can also induce appressorium formation in Uromyces (Hoch and Staples, 1984). Thus, there are shared features in the signaling pathways controlling appressorium development in fungi.

1.2.2 SEX PHEROMONES

Sex pheromones are signal molecules that direct the developmental processes in most eukaryotic organisms. Disruption of pheromone signaling has become an important tool for the control of insects, but similar uses of pheromones have not been investigated for plant disease control. Pheromones are known to be produced by fungi, and in the yeast they have been intensively studied as a model for regulation of development. Little is known, however, about pheromones of filamentous ascomycetes.

Biological control of plant pathogens has lagged that of insects. In part this has been the consequence of focus of efforts of plant pathologists on a different spectrum of biological control strategies. For instance, the use of pheromones and other semiochemicals, while receiving much attention by entomologists, is an area of research that has been neglected by plant pathologists. Signaling between host and pathogen, of course, is currently a research area of intense interest in plant pathology, but, although microbes are known to regulate developmental processes through release of signal chemicals, little attention has been directed toward the use of such chemical signals for plant disease control.

The most common approach for using pheromones to control insects is disruption of mating by perturbing normal pheromone signaling by changing timing, locations, and/or concentrations of naturally occurring pheromones (Jutsum and Gordon, 1989).

1.3 DEFICIENCIES IN THE PRIOR ART

What is lacking in the prior art, therefore, is the identification of DNA segments encoding fungal pheromones, and compositions comprising pheromone polypeptides which inhibit fungal growth and development, and which prevent or reduce fungal infection and/or disease in plant host cells. Also lacking are methods and processes utilizing fungal pheromones and pheromone derivatives in the prevention and treatment of fungal colonization of a plant.

2. SUMMARY OF THE INVENTION

The present invention seeks to overcome these and other inherent deficiencies in the prior art by providing compositions comprising novel polypeptides which are used to prevent fungal infection and disease in plant cells.

The inventors have explored fungal mating disruption as a means for plant disease control, and particularly the use of fungal sex pheromones, or their derivatives, to control plant disease and fungal infection. Many diseases caused by ascomycetes depend upon ascospores for primary inoculum. Reduction of this primary inoculum is a key strategy for the control of these diseases. Thus, disruption of sexual reproduction of ascomycetes provides an important new tool for plant

disease control.

The present invention provides methods and compositions for preventing fungal pathogens from causing disease in plants. The invention concerns the use of pheromones and pheromone derivatives to alter, prevent, diminish, reduce or interfere with growth and/or development and differentiation of fungi and fungal spores, and to reduce or prevent the pathogenicity of fungal species to plant cells.

The present invention provides methods and compositions for preventing or interfering with fungal development. In particular, methods are provided for inhibition of appressorium formation, and for conidial germination of a fungus. In preferred embodiments, the fungus is a pathogenic fungus, and in particular, a plant pathogen.

In an important embodiment, the invention provides novel methods and compositions comprising pheromone receptor polypeptides as targets for interfering with fungal pathogenesis in plant cells

In one embodiment, the inventors have demonstrated the ability of pure α-factor pheromone of *Saccharomyces cerevisiae* to inhibit appressorium formation of *Magnaporthe grisea* rice pathogen strain, Guy11, and the barley pathogen strain, 4091-5-8, *in vitro*. These strains are both mating type MAT1-2 (the two mating types of *M. grisea* are termed MAT1-1 and MAT1-2). The ability of *S. cerevisiae* α-factor pheromone to block infection of barley plants by 4091-5-8 was also demonstrated.

Another aspect of the invention is a composition comprising a purified yeast factor which inhibits appressorium formation in *M. grisea* in a mating type specific manner similar to α-factor.

A further aspect of the invention is a composition comprising a novel pheromone isolated and purified from *M. grisea* mating type MAT1-1 strains. Methods are provided for utilizing this pheromone to block appressorium formation of strains of mating type MAT1-2.

In another embodiment, the invention provides a composition comprising a novel pheromone isolated and purified from *Cryphonectria parasitica*. Methods are also provided for utilizing this pheromone to inhibit growth of germinating conthis fungus. Methods are also disclosed for the use of these and related fungal pheromones to alter multiple stages of fungal growth and development.

In a further embodiment, the invention concerns the preparation of synthetic pheromone compositions which have been shown to control the growth and infectivity of filamentous fungi, to disrupt fungal mating, inhibit growth, and prevent conidiation. In an exemplary embodiment, a synthetic pheromone composition was used to control development of *C. parasitica*.

The inventors have also shown that the mating pheromone receptor provides a novel target for therapeutic agents, and that mating pheromones represent anti-pathogenicity agents which may also be used to screen for additional inhibitory agents.

C. parasitica is the first filamentous fungus from which all mating pheromone genes have been cloned and for which pure mating pheromone peptide has been tested for biological activity. As such, the biological activities discovered for this pheromone are the first demonstration of the role of a pheromone in filamentous ascomycete fungi, and further demonstrate the concept that pheromones could be used to block pathogenic development.

In another embodiment, the invention provides a novel target (a pheromone receptor) for disease control and identifies compounds that act at the target (mating pheromones). These pheromone factors are composed of peptides and/or lipid modified peptides that are natural products that are expected to be harmless to humans, plants, and animals, and such proteins are readily degraded in the environment. Their structures may be modified to alter their potency and their persistence in the environment for optimal disease control and safe use in the environment.

The invention provides a transgenic plant, the genome of which has been augmented through the introduction of a fungal pheromone gene, wherein the gene confers to the plant resistance to fungal infection. Alternatively, the gene confers to the plant the ability to inhibit appressorium formation or conidial maturation or differentation of a fungal cell. Preferably, the fungal pheromone gene is transmittable through normal sexual reproduction of the transgenic plants to subsequent generation plants, wherein the offspring or progeny of the transformed plant inherits the fungal resistance conferred by *in planta* expression of the transgene. In certain embodiments, the pheromone is a mating-type specific pheromone.

In certain embodiments, one or more fungal pheromone-encoding genes are positioned under the control of one or more inducible or tissue-specific promoters or enhancers, as described herein, to direct the expression of the pheromone genes. These genes may be present on a single vector which may be transformed into a recipient plant cell culture. Alternatively the fungal genes may be contained on separate vectors which may be used to simultaneously or sequentially cotransform a suitable recipient cell culture. These vectors may further comprise one or more genetic elements selected from the group consisting of a promoter, an enhancer, a 5' non-coding region, and a 3' non-coding region. Exemplary promoters envisioned to be useful for expression of the fungal genes in a plant host cell include the CaMV 35S, CaMV 19S, *nos*, Adh, sucrose synthase, R-allele and root cell promoters, which are well-known in the art.

Preferred transgenes for use in construction of transgenic plants include genes which encode a protein or peptide selected from the group consisting of Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 and MFBβ1.

In certain embodiments, it may be desirable to alter the coding sequence (or "plantize") the pheromone transgene prior to transformation to improve expression of the gene in the plant host cell. Methods for mutagenizing and altering DNA segments are known to those of skill in the art, and discussed in detail, herein. Generally, the mutagenesis of a transgene will involve random mutagenesis, transposon mutagenesis, site-directed mutagenesis, nucleotide addition or deletion, truncation, or gene fusion techniques.

The transgenic plants of the invention are generally preparable by a process comprising the steps of: (a) obtaining a DNA composition which comprises one or more fungal pheromone genes; (b) contacting recipient plant cells with the DNA composition; (c) regenerating plants from recipient cells which have received the DNA composition; and (d) identifying a fertile, transgenic plant whose genome has been augmented relative to that of the corresponding nontransgenic recipient cells through the stable introduction of the DNA composition.

In an exemplary embodiment, a process is provided for producing a fertile transgenic plant resistant to fungal infection. The process generally involves the steps of

(i) establishing a regenerable culture from a plant to be transformed, (ii) transforming the culture with a DNA composition comprising a fungal pheromone gene, (iii) identifying or selecting a transformed cell line and (iv) regenerating a fertile transgenic plant therefrom. Preferably, the fungal pheromone gene is transmitted through a complete sexual cycle of the transgenic plant to its progeny, wherein the progeny comprises the fungal pheromone gene, and wherein the fungal pheromone gene is chromosomally integrated in the genomes of both the parent and its progeny.

A method of using a DNA segment to identify the presence of a transgene in a sample, and particularly in a polynucleotide sample obtained from a transformed host cell, is also one aspect of the invention. The method generally comprises the steps of: (a) obtaining sample nucleic acids suspected of encoding a fungal pheromone protein; (b) contacting the sample nucleic acids with an isolated nucleic acid segment encoding the pheromone protein under conditions effective to allow hybridization of substantially complementary nucleic acids; and (c) detecting the hybridized complementary nucleic acids thus formed.

A method of using a DNA segment to prepare recombinant pheromone proteins or peptides is also provided by the invention. The method generally comprises the steps of: (a) preparing a recombinant vector in which a fungal pheromone protein or peptide-encoding DNA segment is positioned under the control of a promoter; (b) introducing the recombinant vector into a host cell; (c) culturing the host cell under conditions effective to allow expression of the encoded fungal pheromone protein or peptide; and (d) collecting the expressed pheromone protein or peptide.

2.1 PHEROMONE COMPOSITIONS

Particularly preferred proteins for the practice of the invention include fungal pheromone or mating type proteins which are isolatable from a fungal cell, and which proteins inhibit, prevent, lessen, or diminish conidial development, differentiation, sporulation, growth, mycelial formation, cellular division, and/or virulence of a fungal cell, and particularly, of a plant fungal pathogen.

Protein compositions contemplated to be useful in the practice of the present invention include, but are not limited to, Vir1 and Vir2 from *Cryphonectria parasitica*, MFα1-1, MFα1 and MFα2 from *S. cerevisiae*, MFα from *C. neoformans*,

P-factor, MFm1 and MFm2 from *S. pombe*, MFα1 and MFα2 from *U. maydis*, MAT1-1 and MAT1-2 from *Cochliobolus heterostrophus*, MFBα1 and MFBβ1 from *Schizophyllum commune*.

In addition to those pheromones described, several recent reports have reviewed the molecular genetics and biochemistry of a variety of fungal pheromones and fungal mating types in detail. Irie *et al.* (1994) have reviewed the mating pheromone signal transduction in yeast, while Kurjan (1992) and Leberer *et al.*(1997) describe general pheromone response and signalling in yeast. Signalling pathways in yeast are also discussed in a review by Reed (1991). A review by Nielsen and Davey (1995) describes pheromone communication in *Schizosaccharomyces pombe*, a fission yeast.

The genetics of mating and development of *Ustilago maydis*, a fungal pathogen which induces tumors in maize, is reviewed in publications by Banuett (1995) and Kahmann *et al.* (1995).

Tetrapolar fungal mating types have been described in a recent communication by Kothe (1996), while Vaillancourt and Raper have recently reviewed pheromone receptors and pheromonte mating-type determinants in basidiomycetes (1996).

2.2 Transgenic Plants

Another aspect of the invention comprises a transgenic plant which express a gene or gene segment encoding one or more of the fungal pheromone polypeptide compositions disclosed herein. As used herein, the term "transgenic plant" is intended to refer to a plant that has incorporated DNA sequences, including but not limited to genes which are perhaps not normally present, DNA sequences not normally transcribed into RNA or translated into a protein ("expressed"), or any other genes or DNA sequences which one desires to introduce into the non-transformed plant, such as genes which may normally be present in the non-transformed plant but which one desires to either genetically engineer or to have altered expression.

It is contemplated that in some instances the genome of a transgenic plant of the present invention will have been augmented through the stable introduction of one or more fungal pheromone-encoding transgenes, either native, synthetically modified, or mutated. In some instances, more than one transgene will be incorporated into the

genome of a single transformed host plant cell. Such is the case when more than one pheromone protein-encoding DNA segment is incorporated into the genome of such a plant. In certain situations, it may be desirable to have one, two, three, four, or even more pheromone proteins (either native or recombinantly-engineered) incorporated and stably expressed in the transformed transgenic plant.

A preferred gene which may be introduced includes, for example, a pheromone protein-encoding a DNA sequence from fungal origin, and particularly one or more of those described herein which are obtained from fungal pathogens in the genera *Alternaria*; *Ascochyta*; *Botrytis*; *Cercospora*; *Cochliobolus*; *Cryphonectria*; *Cryptococcus*; *Colletotrichum*; *Diplodia*; *Erysiphe*; *Fusarium*; *Gaeumanomyces*; *Helminthosporium*; *Macrophomina*; *Magnaporthe*; *Nectria*; *Peronospora*; *Phoma*; *Phymatotrichm*; *Phytophthora*; *Plasmopara*; *Podosphaera*; *Puccinia*; *Puthium*; *Pyrenophora*; *Pyricularia*; *Pythium*; *Rhizoctonia*; *Saccharomyces*; *Scerotium*; *Schizophyllum*; *Schizosaccharomyces*, *Sclerotinia*; *Septoria*; *Thielaviopsis*; *Uncinula*; *Ustilago*; *Venturia*; *and Verticillium*.

Highly preferred nucleic acid sequences are those obtained from *Magnaporthe grisea*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Cryptococcus neoformans*, *Schizophyllum commune*, *Ustilago maydis*, *and Cryphonectria parasitica*, or any of those sequences which have been genetically engineered to decrease or increase the antifungal activity of the pheromone protein in such a transformed host cell. Also preferred are nucleic acid sequences encoding fungal pheromones which hybridize to a gene encoding one of these pheromones.

Such transgenic plants may be desirable for increasing the fungal resistance of a monocotyledonous or dicotyledonous plant, by incorporating into such a plant, a transgenic DNA segment encoding one or more fungal pheromones which are toxic to fungi.

Particularly preferred plants include fiber crops such as flax and cotton, grain crops such as corn, wheat, oats, barley, rye, and rice; leap crops such as tobacco; grasses, such as turf and pasture grasses; vegetables; fruits and berries; ornamentals, including house plants, fruit trees, and the like.

In a related aspect, the present invention also encompasses a seed produced by the transformed plant, a progeny from such seed, and a seed produced by the progeny

of the original transgenic plant, produced in accordance with the above process. Such progeny and seeds will have a pheromone protein-encoding transgene stably incorporated into their genome, and such progeny plants will inherit the traits afforded by the introduction of a stable transgene in Mendelian fashion. All such transgenic plants having incorporated into their genome transgenic DNA segments encoding one or more fungal pheromone proteins or polypeptides are aspects of this invention.

All such transgenic plants having incorporated into their genome transgenic DNA segments encoding one or more pheromone proteins or polypeptides (and particularly those proteins disclosed in FIG. 1 and Section 5 herein) are aspects of this invention. Particularly preferred proteins for the practice of the invention include Vir1 and Vir2 from *Cryphonectria parasitica*, MFα1-1, MFα1 and MFα2 from *S. cerevisiae*, MFα from *C. neoformans*, P-factor, MFm1 and MFm2 from *S. pombe*, MFα1 and MFα2 from *U. maydis*, MAT1-1 and MAT1-2 from *Cochliobolus heterostrophus*, MFBα1 and MFBβ1 from *Schizophyllum commune*.

Particularly preferred transgenes are nucleic acid segments comprising one or more of the mating pheromones described herein, and in particular, DNA segments which comprise *Cryphonectria parasitica Vir1* and *Vir2* genes; *S. cerevisiae MFα1-1*, *MFα1* and *MFα2* genes; *C. neoformans MFα* genes; genes encoding the P-factor, MFm1 and MFm2 pheromones of *S. pombe*; genes encoding the MFα1 and MFα2 pheromones of *Ustilago maydis*; *Cochliobolus heterostrophus MAT1-1* and *MAT1-2* genes, as well as *Schizophyllum commune MFBα1* and *MFBβ1* gene(s). Also contemplated to be useful in the practice of the present invention are genes encoding a fungal pheromone regardless of the species from which such a gene is identified and isolated, so long as such a gene, when provided to a plant confers to the plant, fungal resistance, or prevents conidial differentiation of fungal cells present on or in said plant cell.

2.3 PHEROMONE COMPOSITIONS AS ANTIFUNGALS

The compositions of the present invention are contemplated to be useful as antifungal compositions for use in the prevention of fungal growth, sporulation, and/or differentiation. The inventors contemplate that the compositions and transgenes described herein will find broad utility against a variety of plant fungal

pathogens, including those of the genera *Alternaria*; *Ascochyta*; *Botrytis*; *Cercospora*; *Cochliobolus; Cryphonectria; Cryptococcus; Colletotrichum*; *Diplodia*; *Erysiphe*; *Fusarium*; *Gaeumanomyces*; *Helminthosporium*; *Macrophomina*; *Magnaporthe; Nectria*; *Peronospora*; *Phoma*; *Phymatotrichm*; *Phytophthora*; *Plasmopara*; *Podosphaera*; *Puccinia*; *Puthium*; *Pyrenophora*; *Pyricularia*; *Pythium*; *Rhizoctonia*; *Saccharomyces; Scerotium*; *Schizophyllum; Schizosaccharomyces, Sclerotinia*; *Septoria*; *Thielaviopsis*; *Uncinula*; *Ustilago; Venturia*; *and Verticillium*.

When the antifungal compositions comprise intact recombinant cells expressing one or more of the fungal pheromone proteins, such cells may be formulated in a variety of ways. They may be employed as wettable powders, granules or dusts, by mixing with various inert materials, such as inorganic minerals (phyllosilicates, carbonates, sulfates, phosphates, and the like) or botanical materials (powdered corncobs, rice hulls, walnut shells, and the like). The formulations may include spreader-sticker adjuvants, stabilizing agents, other fungicidal additives, or surfactants. Liquid formulations may be aqueous-based or non-aqueous and employed as foams, suspensions, emulsifiable concentrates, or the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers.

Alternatively, the pheromone proteins may be prepared by native or recombinant bacterial or fungal expression systems *in vitro* and isolated for subsequent field application. Such protein may be either in crude cell lysates, mycelial cultures, suspensions, colloids, *etc.*, or alternatively may be purified, refined, buffered, and/or further processed, before formulating in an active antifungal formulation. Likewise, under certain circumstances, it may be desirable to isolate the pheromones from a bacterial or fungal culture expressing the pheromone protein and apply solutions, suspensions, or collodial preparations of such pheromones as the active antifungal composition.

Regardless of the method of application, the amount of the active component(s) is applied at an antifungally-effective amount, which will vary depending on such factors as, for example, the specific fungi to be controlled, the specific plant or crop to be treated, the environmental conditions, and the method, rate, and quantity of application of the antifungally-active composition.

The antifungal compositions described may be made by formulating either the bacterial or fungal cell, pheromone and/or cell suspension, or isolated protein component with the desired agriculturally-acceptable carrier. The compositions may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, dessicated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, inert components, dispersants, surfactants, tackifiers, binders, *etc.* that are ordinarily used in agricultural formulation technology; these are well known to those skilled in antifungal formulation. The formulations may be mixed with one or more solid or liquid adjuvants and prepared by various means, by homogeneously mixing, blending and/or grinding the antifungal composition with suitable adjuvants using conventional formulation techniques.

The antifungal compositions of this invention are applied to the environment of the target fungal pest, typically onto the foliage of the plant or crop to be protected, by conventional methods, preferably by spraying. The strength and duration of antifungal application will be set with regard to conditions specific to the particular pest(s), crop(s) to be treated and particular environmental conditions. The proportional ratio of active ingredient to carrier will naturally depend on the chemical nature, solubility, and stability of the antifungal composition, as well as the particular formulation contemplated.

Other application techniques, including dusting, sprinkling, soaking, soil injection, seed coating, seedling coating, spraying, aerating, misting, atomizing, and the like, are also feasible and may be required under certain circumstances such as *e.g.*, fungi that cause root or stalk infection, or for application to delicate vegetation or ornamental plants. These application procedures are also well-known to those of skill in the art.

The antifungal compositions of the invention may be employed in the method of the invention singly or in combination with other compounds, including and not

limited to other antifungal compositions, insecticidal compositions, fertilizers, antibiotics, or antibacterials. The method of the invention may also be used in conjunction with other treatments such as surfactants, detergents, polymers or time-release formulations. The antifungal compositions of the present invention may be formulated for either systemic or topical use.

The concentration of antifungal compositions which is used for environmental, systemic, or foliar application will vary widely depending upon the nature of the particular formulation, means of application, environmental conditions, and degree of antifungal activity. Typically, the antifungal composition will be present in the applied formulation at a concentration of at least about 1% by weight and may be up to and including about 99% by weight. Dry formulations of the pheromone protein compositions may be from about 1% to about 99% or more by weight of the protein composition, while liquid formulations may generally comprise from about 1% to about 99% or more of the active ingredient by weight. Formulations which comprise intact fungal or bacterial cells which express the pheromone compositions will generally contain from about 10^3 to about 10^{10} cells/mg.

The antifungal formulations may be administered to a particular plant or target area in one or more applications as needed. A typical field application rate per hectare may range on the order of from about 50 g to about 500 g of active ingredient, or of from about 500 g to about 1000 g, or of from about 1000 g to about 5000 g or more of active ingredient. The particular amount of the preparation required for a particular application will be readily quantitatable by one of skill in the art, depending upon the specific application, the level of antifungal activity required, and the activity of the particular pheromone preparation utilized.

2.4 GENETIC ANALYSIS OF TRANSGENIC PLANTS

Methods may be used for detecting the presence or expression of the transgenes of the current invention. The method of assaying expression may comprise determining the level of protein expressed by the transgene or by determining specific alterations in the expressed product. Such assays may in some cases be faster, more accurate or less expensive than conventional screening assays.

The biological sample may potentially be any type of plant tissue. Nucleic

acid may be isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

Following detection, one may compare the results seen in a given plant with a statistically significant reference group of non-transformed control plants. Typically, the non-transformed control plants will be of a genetic background similar to the transformed plants. In this way, it is possible to detect differences in the amount or kind of protein detected in various transformed plants.

A variety of different assays are contemplated in the screening of transgenic plants created using the methods of the current invention. These techniques can be used to detect both the presence of particular genes as well as rearrangements that may have occurred in the gene construct. The techniques include but are not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA), RNAse protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR™-SSCP.

2.5 PHEROMONE-ENCODING DNA SEGMENTS

The present invention also concerns DNA segments that can be isolated from virtually any source, that are free from total genomic DNA and that encode fungal pheromones which have antifungal activity *in vitro* and/or *in vivo*. DNA segments encoding these peptide species may prove to encode proteins, polypeptides, subunits,

functional domains, and the like of pheromone-related or other non-related gene products. In addition these DNA segments may be synthesized entirely *in vitro* using methods that are well-known to those of skill in the art.

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a pheromone protein or peptide refers to a DNA segment that contains pheromone protein coding sequences yet is isolated away from, or purified free from, total genomic DNA of the fungal species from which the DNA segment is obtained. Particularly preferred DNAs are those encoding pheromones isolatable from the genome of a fungus such as *S. cerevisiae, M. grisea,* or *C. parasitica.* Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified pheromone-encoding gene refers to a DNA segment which may include in addition to peptide encoding sequences, certain other elements such as, regulatory sequences, isolated substantially away from other naturally occurring genes or protein-encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein-, polypeptide- or peptide-encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, operon sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding a bacterial pheromone protein, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or operon coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes, recombinant genes, synthetic linkers, or coding regions later added to the segment by the hand of man.

The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein (*e.g.*, see Illustrative Embodiments). Accordingly,

sequences that have between about 70% and about 80%, or more preferably between about 81% and about 90%, or even more preferably between about 91% and about 99% amino acid sequence identity or functional equivalence to the amino acids of the fungal pheromone sequences disclosed herein are particularly preferred.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5′ or 3′ sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5′ or 3′ portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch encoding either of the peptide sequences disclosed, or that are identical to or complementary to DNA sequences which encode any of the peptides disclosed herein. For example, DNA sequences such as about 18 nucleotides, and that are up to about 10,000, about 5,000, about 3,000, about 2,000, about 1,000, about 500, about 200, about 100, about 50, and about 14 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 18, 19, 20, 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; and up to and including sequences of about 6000, 7000, 8000, 9000, and

10,000 nucleotides and the like.

It will also be understood that this invention is not limited to the particular nucleic acid sequences which encode peptides of the present invention. Recombinant vectors and isolated DNA segments may therefore variously include the peptide-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include these peptide-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically-functional, equivalent peptides. Such sequences may arise as a consequence of codon degeneracy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally-equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the molecular level.

If desired, one may also prepare fusion proteins and peptides, *e.g.*, where the peptide-coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a gene encoding peptides of the present invention, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

2.6 DNA Segments as Hybridization Probes and Primers

In addition to their use in directing the expression of pheromone proteins or peptides of the present invention, the nucleic acid sequences contemplated herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous DNA segment of a gene encoding a fungal pheromone disclosed in SEQ ID NO:1-SEQ ID NO:16 will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000, 2000, 5000 bp, *etc.* (including all intermediate lengths and up to and including the full-length sequence of 5200 basepairs will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to pheromone protein-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to a DNA sequence encoding a fungal pheromone disclosed in any of SEQ ID NO:1-SEQ ID NO:16, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 or 200 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated

oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patents 4,683,195 and 4,683,202 (each incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating pheromone protein-encoding DNA segments. Detection of DNA segments via hybridization is well-known to those of skill in the art, and the teachings of U. S. Patents 4,965,188 and 5,176,995 (each incorporated herein by reference) are exemplary of the methods of hybridization analyses. Teachings such as those found in the texts of Maloy *et al.*, 1993; Segal, 1976; Prokop, 1991; and Kuby, 1991, are particularly relevant.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate pheromone protein-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as

increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

2.7 METHODS FOR RECOMBINANT EXPRESSION OF PHEROMONE GENES

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a DNA segment encoding a pheromone protein or peptide in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters

isolated from any bacterial, viral, eukaryotic, or plant cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology).

In connection with expression embodiments to prepare recombinant proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire peptide sequence being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of pheromone peptides or epitopic core regions, such as may be used to generate anti-pheromone protein antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 8 to about 50 amino acids in length, or more preferably, from about 8 to about 30 amino acids in length, or even more preferably, from about 8 to about 20 amino acids in length are contemplated to be particularly useful. Such peptide epitopes may be amino acid sequences which comprise contiguous amino acid sequences from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO1:4, SEQ ID NO:15, or SEQ ID NO:16.

2.8 Design of Synthetic Plant Genes

For optimum expression of particular transgenes in plants, it may be desired to adjust the DNA sequence of the transgene to more closely resemble that of genes which are efficiently expressed in the host plant. Such replacement may be carried out by substitution of bases in a native gene sequence without changing the sequence of the encoded polypeptide. In this manner, an identical polypeptide may be

produced, but at levels much higher than would otherwise be produced using constructs comprising the native gene sequence. Such alterations may be particularly desirable in the case of genes which were originally isolated from organisms distantly related to the plant host, such as with the case of bacterial genes.

In designing synthetic genes for enhanced expression in plants, the DNA sequence of the native structural gene is modified in order to contain codons preferred by highly expressed plant genes. Preferably, the A+T content of the synthetic gene is substantially equal to that of genes for highly expressed proteins in the host plant. In genes encoding highly expressed plant proteins, the A+T content is approximately 55%. It is preferred that the synthetic gene have an A+T content near this value, and not sufficiently high as to cause destabilization of RNA and, therefore, lower the protein expression levels. More preferably, the A+T content is no more than about 60% and most preferably is about 55%.

It is known in the art that seldom-used codons are generally detrimental to gene expression and must be avoided or used judiciously. Thus, in designing a synthetic gene encoding the desired transgene polypeptide, individual amino acid codons found in the original gene are altered to reflect the codons preferred by the host plant for a particular amino acid. However, attention is given to maintaining the overall distribution of codons for each amino acid within the coding region of the gene. Hence, the synthetic gene is designed such that its distribution frequency of codon usage deviates, preferably, no more than 25% from that of highly expressed plant genes and, more preferably, no more than about 10%. The preferred codons for design of synthetic genes for expression in particular plants will be well known to those of skill in the art.

In designing a heterologous gene for expression in plants, sequences which interfere with the efficacy of gene expression, such as plant polyadenylation signals, polymerase II termination sequences, hairpins, plant consensus splice sites and the like, are eliminated. Also, for ultimate expression in plants, the synthetic gene nucleotide sequence is preferably modified to form a plant initiation sequence at the 5' end of the coding region. In addition, particular attention is preferably given to assure that unique restriction sites are placed in strategic positions to allow efficient assembly of oligonucleotide segments during construction of the synthetic gene and to

facilitate subsequent nucleotide modification. Consideration will also be given to the percentage G+C content of the degenerate third base (monocot plants appear to favor G+C in this position, whereas dicots do not). It is recognized that the XCG nucleotide is the least preferred codon in dicots whereas the XTA codon is avoided in both monocots and dicots. Synthetic genes will also preferably have CG and TA doublet avoidance indices approximating those of the chosen host plant.

As is known to those skilled in the art of synthesizing genes (Mandecki *et al.* (1985) Proc. Natl. Acad. Sci. 82:3543-3547; Feretti *et al.* (1986) Proc. Natl. Acad. Sci. 83:599-603), the DNA sequence to be synthesized is divided into segment lengths which can be synthesized conveniently and without undue complication. Each segment has unique restriction sequences at the cohesive ends. Single-stranded oligonucleotides are annealed and ligated to form the DNA segments. The length of the protruding cohesive ends in complementary oligonucleotide segments is four to five residues. In the strategy evolved for gene synthesis, the sites designed for the joining of oligonucleotide pieces and DNA segments are different from the restriction sites created in the gene. The nucleotide sequence of each fragment is determined at this stage by the dideoxy method using recombinant phage DNA as templates and selected synthetic oligonucleotides as primers. Each segment individually is excised at the flanking restriction sites from its cloning vector and spliced into the vector-containing segment. Most often, segments are added as a paired segment instead of as a single segment to increase efficiency. In this way, the entire gene is constructed in the original plasmid. Exemplary procedures for the design and production of synthetic genes which may be used in the context of the current invention are disclosed in, for example, U. S. Patents 5,567,600; 5,567,862; and 5,508,468; each of which is specifically incorporated herein by reference in its entirety.

2.9 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes may be made in the structure of the pheromone peptides of the present invention and DNA segments which encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule.

In particular embodiments of the invention, mutated pheromone proteins are contemplated to be useful for increasing the antifungal activity of the protein, and consequently increasing the antifungal activity and/or expression of the recombinant transgene in a plant cell. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the codons given in Table 1.

TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites

on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological

property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

2.10 Peptide and Peptide Derivative Compositions

The present invention is also directed to protein or peptide compositions, free from total cells and other peptides, which comprise a purified peptide which incorporates an epitope that is immunologically cross-reactive with one or more anti-pheromone protein antibodies. In particular, the invention also concerns truncated peptides, peptide fusions, active site regions, amino-terminal and carboxy-terminal fragments, and other peptides derived from a fungal pheromone proteins.

As used herein, the term "incorporating an epitope(s) that is immunologically cross-reactive with one or more anti-pheromone protein antibodies" is intended to refer to a peptide or protein antigen which includes a primary, secondary or tertiary structure similar to an epitope located within a pheromone protein or polypeptide. The level of similarity will generally be to such a degree that monoclonal or

polyclonal antibodies directed against the pheromone protein or polypeptide will also bind to, react with, or otherwise recognize, the cross-reactive peptide or protein antigen. Various immunoassay methods may be employed in conjunction with such antibodies, such as, for example, Western blotting, ELISA, RIA, and the like, all of which are known to those of skill in the art.

The identification of immunodominant epitopes, and/or their functional equivalents, suitable for use in vaccines is a relatively straightforward matter. For example, one may employ the methods of Hopp, as taught in U. S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. The methods described in several other papers, and software programs based thereon, can also be used to identify epitopic core sequences (see, for example, Jameson and Wolf, 1988; Wolf *et al.*, 1988; U. S. Patent 4,554,101). The amino acid sequence of these "epitopic core sequences" may then be readily incorporated into peptides, either through the application of peptide synthesis or recombinant technology.

Preferred peptides for use in accordance with the present invention will generally be on the order of about 8 to about 20 amino acids in length, and more preferably about 8 to about 15 amino acids in length. It is proposed that shorter antigenic pheromone protein-derived peptides will provide advantages in certain circumstances, for example, in the preparation of immunologic detection assays. Exemplary advantages include the ease of preparation and purification, the relatively low cost and improved reproducibility of production, and advantageous biodistribution.

It is proposed that particular advantages of the present invention may be realized through the preparation of synthetic peptides which include modified and/or extended epitopic/immunogenic core sequences which result in a "universal" epitopic peptide directed to one or more of the pheromone proteins of the present invention. These epitopic core sequences are identified herein in particular aspects as hydrophilic regions of the particular polypeptide antigen. It is proposed that these regions represent those which are most likely to promote T-cell or B-cell stimulation, and, hence, elicit specific antibody production.

An epitopic core sequence, as used herein, is a relatively short stretch of amino

acids that is "complementary" to, and therefore will bind, antigen binding sites on the pheromone-specific antibodies disclosed herein. Additionally or alternatively, an epitopic core sequence is one that will elicit antibodies that are cross-reactive with antibodies directed against the peptide compositions of the present invention. It will be understood that in the context of the present disclosure, the term "complementary" refers to amino acids or peptides that exhibit an attractive force towards each other. Thus, certain epitope core sequences of the present invention may be operationally defined in terms of their ability to compete with or perhaps displace the binding of the desired protein antigen with the corresponding protein-directed antisera.

In general, the size of the polypeptide antigen is not believed to be particularly crucial, so long as it is at least large enough to carry the identified core sequence or sequences. The smallest useful core sequence anticipated by the present disclosure would generally be on the order of about 8 amino acids in length, with sequences on the order of 10 to 20 being more preferred. Thus, this size will generally correspond to the smallest peptide antigens prepared in accordance with the invention. However, the size of the antigen may be larger where desired, so long as it contains a basic epitopic core sequence.

The identification of epitopic core sequences is known to those of skill in the art, for example, as described in U. S. Patent 4,554,101, incorporated herein by reference, which teaches the identification and preparation of epitopes from amino acid sequences on the basis of hydrophilicity. Moreover, numerous computer programs are available for use in predicting antigenic portions of proteins (see *e.g.*, Jameson and Wolf, 1988; Wolf *et al.*, 1988). Computerized peptide sequence analysis programs (*e.g.*, DNAStar® software, DNAStar, Inc., Madison, WI) may also be useful in designing synthetic peptides in accordance with the present disclosure.

Syntheses of epitopic sequences, or peptides which include an antigenic epitope within their sequence, are readily achieved using conventional synthetic techniques such as the solid phase method (*e.g.*, through the use of commercially available peptide synthesizer such as an Applied Biosystems Model 430A Peptide Synthesizer). Peptide antigens synthesized in this manner may then be aliquotted in predetermined amounts and stored in conventional manners, such as in aqueous solutions or, even more preferably, in a powder or lyophilized state pending use.

In general, due to the relative stability of peptides, they may be readily stored in aqueous solutions for fairly long periods of time if desired, *e.g.*, up to six months or more, in virtually any aqueous solution without appreciable degradation or loss of antigenic activity. However, where extended aqueous storage is contemplated it will generally be desirable to include agents including buffers such as Tris or phosphate buffers to maintain a pH of about 7.0 to about 7.5. Moreover, it may be desirable to include agents which will inhibit microbial growth, such as sodium azide or Merthiolate. For extended storage in an aqueous state it will be desirable to store the solutions at about 4°C, or more preferably, frozen. Of course, where the peptides are stored in a lyophilized or powdered state, they may be stored virtually indefinitely, *e.g.*, in metered aliquots that may be rehydrated with a predetermined amount of water (preferably distilled) or buffer prior to use.

2.11 PEPTIDE MIMETIC COMPOSITIONS

Another embodiment for the preparation of polypeptides according to the present invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *Biotechnology and Pharmacy*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule. These principles may be used, in conjunction with the principles outline above, to engineer second generation molecules having many of the natural properties of the original molecule, but with altered and even improved characteristics.

2.12 FUSION PROTEINS

A specialized kind of insertional variant is the fusion protein. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a

protein in a heterologous host. Another useful fusion includes the addition of a immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, or cellular targeting signals. Fusion to a polypeptide that can be used for purification of the substrate-protein complex could be used to further study the substrate-enzyme interaction.

2.13 SITE-SPECIFIC MUTAGENESIS

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double

stranded vector which includes within its sequence a DNA sequence which encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the pheromone-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

3. BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Sequences of prenylated pheromones from various fungi. The CAAX terminal sequence is a post-translational signal for prenylation. The asparagine in bold indicates a predicted peptide cleavage site involved in pheromone maturation, based upon the pheromones of *S. cerevisiae* and *S. pombe* (figure adapted from Moore and Edman, 1993).

FIG. 2. Comparison of the open reading frames of genes encoding the a-mating type pheromone precursor gene of *C. parasitica* (MTSa1) and that of mating factor-α (MFα1) precursor of *S. cerevisiae*. The Kex2 endopeptidase cleavage sites are shown (KR), the Kex1 cleavage sites (XA(P)) and glycosylation signals (NIT,

NIS, NTT, NST).

FIG. 3. Pheromone binding response pathway of *S. cerevisiae*. Where gene names are provided, the genes are common to both mating types.

FIG. 4A.-FIG. 4H. Yeast α-factor pheromone functions in a mating type-specific manner to repress appressorium formation of *M. grisea*. *M. grisea* conidia were harvested as described. Conidia were quantitated to a final concentration of 1×10^4 conidia/ml. Conidia of *MAT1-2* strain 4091-5-8 (FIG. 4A, FIG. 4B, FIG. 4C, FIG. 4D, and FIG 4H) and *MAT1-1* strain CP987 (FIG. 4E, FIG. 4F, and FIG. 4G) were inoculated onto the hydrophobic side of GelBond film (FMC Biochemicals, ME) in the following solutions: FIG. 4A: 10 mM phosphate buffer, pH 6.5; FIG. 4B and FIG. 4E: 2% yeast extract; FIG. 4C and FIG. 4F: 0.5 mg/ml yeast α-factor; FIG. 4D and FIG. 4G: benzyl alcohol fraction of *M. grisea* strain CP987 culture filtrate; FIG. 4H: 0.5 mg/ml α-factor and 10 mM cAMP. Conidia were incubated for 16 hr and examined by bright field microscopy at 100×. Conidia are approximately 10 μm in length.

FIG. 5A.-FIG. 5D. Yeast pheromone functions in *planta* to prevent pathogenesis of *M. grisea*. *In vivo* assays were performed as follows: ten day old barley seedlings (*Hordeum vulgare* cv. Bonanza) were inoculated with the 3 ml of either 1% gelatin (FIG. 5A; negative control); 1% gelatin with 10^4 conidia per ml. (FIG. 5B); 0.5 mg/ml α-factor pheromone dissolved in 1% gelatin with 10^4 conidia per ml (FIG. 5C); or 0.5 mg/ml control peptide (CQSMSGPAGSPGLLNLIPVDLS-NH_2, SEQ ID NO:15) (FIG. 5D) in 1% gelatin with 10^4 conidia per ml. Seedlings were aerosol spray inoculated and incubated in clear plastic bags overnight to maintain high humidity. The following day, plants were removed from bags and symptoms were allowed to develop for seven days prior to scoring. A second study using the same protocol was subsequently performed with 14-day-old barley seedlings. Lesions were scored with the aid of Image Pro Plus (Media Cybernetics, Silver Springs, MD) to count and measure lesions. Statview (Abacus concepts, Berkeley CA) was used for data analysis.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

4.1 SOME ADVANTAGES OF THE INVENTION

The fungal pheromone proteins disclosed herein are useful in preventing fungal infection, colonization, or infestation in a plant transformed with one or more genes encoding these pheromones.

Synthetic pheromones have been shown to inhibit conidia (asexual spore) of both mating types at germination at a concentration of 10^{-6} M and higher. Synthetic pheromones have also been shown to inhibit conidia of the opposite mating type at a concentration of 10^{-9} M and higher. These synthetic peptides appear to irreversibly inhibit the germination of conidia rather than just temporarily suspend their germination. Importantly, it has been shown that Kex2p processed peptides such as the fungal pheromones described herein can be expressed, processed, and secreted by plants, thereby permitting the use in the development of transgenic plants which express the proteins and are resistant to fungi which are inhibited by the expression of the encoded pheromones.

4.2 DEFINITIONS

The following words and phrases have the meanings set forth below.

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

Regeneration: The process of growing a plant from a plant cell (*e.g.*, plant protoplast or explant).

Structural gene: A gene that is expressed to produce a polypeptide.

Transformation: A process of introducing an exogenous DNA sequence (*e.g.*, a vector, a recombinant DNA molecule) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

Transformed cell: A cell whose DNA has been altered by the introduction of an exogenous DNA molecule into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell. Exemplary transgenic cells include plant calli derived from a transformed plant cell and particular cells such as leaf, root, stem, *e.g.*, somatic cells, or reproductive (germ) cells obtained from a transgenic plant.

Transgenic plant: A plant or progeny thereof derived from a transformed plant cell or protoplast, wherein the plant DNA contains an introduced exogenous DNA molecule not originally present in a native, non-transgenic plant of the same strain. The terms "transgenic plant" and "transformed plant" have sometimes been used in the art as synonymous terms to define a plant whose DNA contains an exogenous DNA molecule. However, it is thought more scientifically correct to refer to a regenerated plant or callus obtained from a transformed plant cell or protoplast as being a transgenic plant, and that usage will be followed herein.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

4.3 PRIMERS AND PROBES

The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable of priming, are designed to binding to the target DNA or RNA and need not be used in an amplification process.

In certain embodiments, the probes or primers may be labeled with radioactive species (^{32}P, ^{14}C, ^{35}S, ^{3}H, or other label), with a fluorophore (rhodamine, fluorescein), an antigen (biotin, streptavidin, digoxigenin), or a chemilumiscent (luciferase) to permit detection and visualization.

4.4 TEMPLATE DEPENDENT AMPLIFICATION METHODS

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U. S. Patents 4,683,195, 4,683,202 and 4,800,159, each of which is specifically incorporated herein by reference in its entirety.

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers dissociate from the marker to form reaction products, excess primers bind to the marker and to the reaction products, and then the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcription of RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases and are described in Int. Pat. Appl. Publ. No. WO 90/07641 (specifically incorporated herein by reference). Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in Eur. Pat. Appl. Publ. No. EP 320308 (specifically incorporated herein by reference in its entirety). In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U. S. Patent 4,883,750 (specifically incorporated herein by reference) describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta® Replicase, described in Int. Pat. Appl. Publ. No. PCT/US87/00880 (specifically incorporated herein by reference), may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992).

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in Great Britain Pat. Appl. No. 2202328 and in Int. Pat. Appl. Publ. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR™-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After

cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, Int. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference in their entirety). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat-denatured again. In either case, the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, Eur. Pat. Appl. Publ. No. EP 329822 (specifically incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of

the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.* (Int. Pat. Appl. Publ. No. WO 89/06700, specifically incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M. A., In: *PCR Protocols: A Guide To Methods And Applications*, Academic Press, New York, 1990; Ohara *et al.*, 1989; each herein incorporated by reference in their entirety).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, (1989), incorporated herein by reference in its entirety.

4.5 SOUTHERN/NORTHERN BLOTTING

Blotting techniques are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provides different types of information, although cDNA blotting is analogous, in many aspects, to blotting or RNA species.

Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter.

Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is

designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

4.6 Separation Methods

It normally is desirable, at one stage or another, to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods. See Sambrook *et al.*, 1989.

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

4.7 Detection Methods

Products may be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by a labeled probe. The techniques involved are well known to those of skill in the art and can be found in many standard books on molecular protocols (see Sambrook *et al.*, 1989). For example, chromophore or

radiolabel probes or primers identify the target during or following amplification.

One example of the foregoing is described in U. S. Patent 5,279,721 (specifically incorporated herein by reference), which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

In addition, the amplification products described above may be subjected to sequence analysis to identify specific kinds of variations using standard sequence analysis techniques. Within certain methods, exhaustive analysis of genes is carried out by sequence analysis using primer sets designed for optimal sequencing (Pignon *et al.*, 1994). The present invention provides methods by which any or all of these types of analyses may be used.

4.8 METHODS FOR RELATIVE QUANTITATIVE RT-PCR™

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR™ (RT-PCR™) can be used to determine the relative concentrations of specific mRNA species isolated from plants. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed.

In PCR™, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR™ amplification is directly proportional to the starting concentration of the target before

the reaction began. By determining the concentration of the amplified products of the target DNA in PCR™ reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundance of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR™ products and the relative mRNA abundance is only true in the linear range of the PCR™ reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundance of a mRNA species can be determined by RT-PCR™ for a collection of RNA populations is that the concentrations of the amplified PCR™ products must be sampled when the PCR™ reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR experiment to successfully determine the relative abundance of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

Most protocols for competitive PCR™ utilize internal PCR™ standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR™ amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundance made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundance of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct

linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR™ assay for plant tissue. The problems inherent in plant tissue samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of larger size than the target). Both of these problems are overcome if the RT-PCR™ is performed as a relative quantitative RT-PCR™ with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR™ assay with an external standard protocol. These assays sample the PCR™ products in the linear portion of their amplification curves. The number of PCR™ cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR™ assays can be superior to those derived from the relative quantitative RT-PCR™ assay with an internal standard.

One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR™ product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR™ product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

4.9 CHIP-BASED DNA TECHNOLOGIES

Specifically contemplated by the present inventors are chip-based DNA technologies such as those described by Hacia *et al.* (1996) and Shoemaker *et al.* (1996). Briefly, these techniques involve quantitative methods for analyzing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ chip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization (see also, Pease *et al.*, 1994; Fodor *et al.*, 1989).

4.10 GENES ENCODING FUNGAL PHEROMONES

Comparisons of pheromone precursor genes that have been isolated from various fungi and the yeasts *S. cerevisiae* and *S. pombe* suggests that pheromone gene structure, transport, and processing is highly conserved in the fungi. Although much is known about pheromone responses of the yeast at both the cellular and molecular levels, some of this information is not directly applicable to the filamentous fungi. For instance, the ascomycetes produce a specialized sexual structure, the ascogonium, that is likely the site of pheromone reception. The yeasts have no such differentiated tissue, so all cells act as ascogonia.

Signaling, such as occurs in the mating of yeast and fungi, is an important and highly conserved developmental process. Because conservation of the general structure of the receptor and the signal transduction pathways extends throughout all eukaryotes, there is much accumulated knowledge concerning these processes (Marsh *et al.*, 1991; Kurjan, 1993; Bardwell *et al.*, 1994; Herskowitz, 1995). In particular, because of its powerful genetics and cellular simplicity, the pheromone signaling process of *S. cerevisiae* has been the focus of much research. Study of receptor homologs to the yeast pheromone receptors has led in mammalian systems to the design of specific drugs that block function of receptors (Strader *et al.*, 1994).

4.11 PHEROMONES OF YEAST AND OTHER FUNGI

The yeast *S. cerevisiae* has two mating types, a and α, each of which produces a mating-type-specific pheromone and a receptor for the pheromones of the other mating type. Precursor genes for the pheromones of both mating types have been

cloned and the native pheromones have been isolated from culture fluids of the two mating types. The a-mating type pheromone is a farnesylated peptide of 12 amino acids; α-mating type pheromone is an unmodified peptide of 13 amino acids that is unrelated in sequence to the a-pheromone. Each is processed from larger translation products and secreted by totally different cellular mechanisms. Both pheromones are hydrophobic, the α-factor being so because of the specific amino acids comprising the tridecapeptide. Two genes encode each of the pheromones. The α-factor precursor genes contain four and three copies respectively of the tridecapeptide encoded in their sequences. Each of the a-pheromone precursor genes encodes a single copy of the pheromone peptide (Marsh *et al.*, 1991).

The pheromone peptide released by one mating type of yeast binds to a specific receptor in the cell membrane of the other mating type. This binding initiates a signal transmission that induces transcription of a number of mating specific genes, the culmination of which is ascospore formation. There are approximately 10,000 α-factor receptor proteins on the surface of each yeast cell (Bardwell *et al.*, 1994). The pheromone receptors of *S. cerevisiae* have seven membrane spanning regions and belong to a well-characterized and conserved class of eukaryotic receptors that include the β-adrenergic and visual rhodopsin receptors. Each member of this class of receptors has seven membrane spanning regions, the internal loops of which are associated with the Gα subunit of a heterotrimeric G-protein (Bardwell *et al.*, 1994).

The simple structure of the yeast pheromones, particularly the α-factor, has facilitated receptor binding studies. Both pheromones have been synthesized and each synthetic product is as active as the native pheromone (Xue *et al.*, 1989; Sherrill *et al.*, 1995). The simplicity of the bioassay for receptor binding of yeast pheromones has also made study of the binding response a simple process. Since one of the first responses to pheromone binding in yeast is an inhibition of growth in G_1 phase, transitory growth inhibition is used as a plate bioassay for pheromone binding. Known amounts of pheromones are added to filter discs on lawns of yeast containing 2×10^6 cells. Growth inhibition, determined as the size of the zone of inhibition, is measured 24 hrs later (Sen and Marsh, 1995). Another effective bioassay of pheromone binding is to fuse *lacZ* to one of the genes (*Fus1*) transcriptionally activated by pheromone binding. In this case, β-galactosidase activity is used as an

assay for pheromone binding (Sen and Marsh, 1995).

Such approaches as these have demonstrated that most changes in the size and sequence of the α-factor result in loss of activity. Studies have also shown that synthetic agonists can bind to the receptor without eliciting a response (Yang *et al.*, 1995). Most studies concerning yeast pheromone receptors focus on requirements of pheromone structure for effective binding, but there is considerable precedent in the literature for design of antagonists as a means to block receptor function (Strader *et al.*, 1994). Such approaches form the basis of modern pharmaceutical research.

Pheromone precursor genes have been described from a few fungi other than *S. cerevisiae*, including *Schizosaccharomyces pombe*, *Cryptococcus neoformans*, *Ustilago maydis*, and *C. parasitica*. In each of these fungi, the pheromone precursor gene(s) encode a short peptide that contains a C-terminal post-translational signal for prenylation of the peptide (FIG. 1). In only a few cases have the pheromones of these fungi been isolated, but in those cases, they have been found to be the farnesylated peptide predicted from the gene sequence. Pheromone precursor genes comparable to the α-factor pheromones of *S. cerevisiae* have been isolated only from *S. pombe*, and *C. parasitica*. The only filamentous ascomycete from which any pheromone precursor genes have been cloned is *C. parasitica*. Information to date about the pheromone precursor gene genes of *C. parasitica* suggests that they have much in common with those of *S. cerevisiae*.

The pheromone precursor genes of *C. parasitica* that correspond to the a-factor pheromones of *S. cerevisiae* were originally isolated as genes that were down-regulated in virus-infected strains (Powell and Van Alfen, 1987). These genes were consequently named *Vir1* and *Vir2* since the transcripts of these genes were present only in virulent, non-virus infected strains. These two genes share a small ORF with a sequence typical of the prenylated fungal pheromones (FIG. 1). Other than this identical ORF, these two genes differ in sequence from each other. The inventors have been able to delete *Vir2* from the genome of *C. parasitica* (Zhang *et al.*, 1993). The phenotype of the null mutant strain was sterility of the fungus, both when used as a male or female parent. There was also, unexpectedly, a 100-fold reduction in asexual sporulation. Additional evidence that *Vir1* and *Vir2* encode a sex pheromone is that these genes are only expressed in mating type Δ of *C. parasitica*. The genes

are present in the other mating type (a) as silent copies. The silent copy of *Vir2* has been sequenced and found to be identical in sequence to that of the expressed gene. In all regards, the A mating type specific pheromone precursor genes of *C. parasitica* appear to follow the model of *S. cerevisiae i.e.* each fungus has two genes encoding a prenylated pheromone and each has silent copies of these genes in the other mating type. FIG. 1 compares the sequences of the ORFs encoding prenylated pheromones of various fungi.

4.12 Pheromone Response Pathway in Yeast

The binding of a pheromone to its receptor initiates a signal that is transmitted from the heterotrimeric G-protein associated with the receptor through a protein kinase cascade culminating in activation of a transcription factor (*Ste12*) that initiates transcription of genes required for mating. Some of the genes activated by *Ste12* include those controlling cell fusion, cell-cycle arrest, and nuclear fusion; additionally, pheromone and receptor transcription is increased in response to pheromone binding. The particular protein kinase cascade associated with the mating response of *S. cerevisiae*, *S. Pombe*, and apparently *U. maydis* is one that is highly conserved in eukaryotes, and is best known as the mitogen-activated protein kinase (MAPK) cascade (Heskowitz, 1995). A diagram describing the components of this signal transduction pathway is shown in FIG. 3.

The inventors have demonstrated that signal-transduction pathways can be activated in yeast by heterologous receptors. The pheromone receptor gene of *S. klyveri*, for instance, has been transformed into *S. cerevisiae* and found to activate its signal transduction pathway after treatment with the pheromones of *S. klyveri* (Marsh and Herskowitz, 1988). Transformation of mammalian receptors into yeast has also been found to successfully result in mating responses if the respective mammalian Gα subunit is included (King *et al.*, 1990). Apparently this mammalian Gα subunit is able to effectively associate with the yeast β and γ subunits to form an active heterotrimeric G-protein capable of transmitting signals.

In these more complex fungi the ascogonium should alone be responsive to pheromones rather than all cells being responsive as with the yeast. Deletion of one of the two MatA specific pheromone precursor genes of *C. parasitica* resulted in a

significant reduction in asexual sporulation (Zhang *et al.*, 1993). When the mutation was complemented, asexual sporulation was restored to normal levels (Zhang *et al.*, 1993). Differences exist in the responses to pheromones among the different yeasts that have been studied, *i.e.* only haploids of *S. cerevisiae* respond to pheromones, but both haploid and dikaryotic cells of *U. maydis* respond to pheromones (Bolker *et al.*, 1992). The a-pheromones of *U. maydis* is continuously required during growth for maintenance of the dikaryon (Banuett and Herskowitz, 1994). In the filamentous fungi, where differentiation of tissues is necessary for sporulation, it is possible that pheromones have evolved a role beyond the initiation of mating.

4.13 Methods for Controlling Fungal Diseases in Plants

There are some inherent advantages of pheromones for plant disease control over their use for insect control. To disrupt mating of insects, pheromones must be dispersed in a large volume of air surrounding the host to be protected (Jutsum and Gordon, 1985; Carde and Minks, 1995). Since the mating structures of the fungi are not mobile, placement of fungal pheromones or their antagonists will be restricted to areas containing the fungal thallus. In the case of chestnut blight, pheromones would only need to be placed on the tree canker. This will obviously reduce the amount of active material applied and should increase the effectiveness of the use of pheromones.

Mating disruption for control of chestnut blight may be important for a number of other diseases caused by ascomycetes. Reduction of ascospore inoculum is a primary strategy for disease control, and reduction of ascospore production on orchard floors would eliminate apple scab as a serious problem and greatly reduce the use of chemical fungicides.

The inventors have cloned the pheromone precursor gene of mating type a of *C. parasitica* by subtractive hybridization between isolates of the fungus that differed in mating. The gene, *Mtsa1*, contains seven repeats of a decapeptide. Associated with these peptides are recognition signals for KEX1 and KEX2 peptidase, the peptidase involved in processing the α-factor of yeast. The *Mtsa1* gene is only expressed in a mating types of *C. parasitica*, although a silent copy can be detected in the other mating type. Only one copy of a gene encoding this decapeptide has been detected in

C. parasitica. In this aspect, *C. parasitica* differs from yeast since *S. cerevisiae* has two genes encoding the α-factor: the two genes of the yeast, however, encode the same number of copies of the peptide sequences as does the single gene of *C. parasitica* (Sprague and Thorner, 1992). FIG. 2 compares the organization of *Mtsa1* with that of the ORF encoding mating factor-α of yeast.

4.14 EXPRESSION VECTORS

The present invention contemplates an expression vector comprising a polynucleotide of the present invention. Thus, in one embodiment an expression vector is an isolated and purified DNA molecule comprising a promoter operatively linked to an coding region that encodes a polypeptide of the present invention, which coding region is operatively linked to a transcription-terminating region, whereby the promoter drives the transcription of the coding region.

As used herein, the term "operatively linked" means that a promoter is connected to an coding region in such a way that the transcription of that coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region are well known in the art.

In one embodiment, the recombinant expression of DNAs encoding the pheromones of the present invention is performed using a transformed Gram-negative bacterium such as an *E. coli* or *Pseudomonas* spp. host cell. Promoters which function in high-level expression of target polypeptides in *E. coli* and other Gram-negative host cells are also well-known in the art.

In another embodiment, the recombinant expression of DNAs encoding the pheromones of the present invention is performed using a transformed fungal or yeast cell, such as a *M. grisea*, *S. cerevisiae*, or *C. parasitica* cell. Promoters which function in high-level expression of target polypeptides in yeast and other fungi are well-known in the art.

4.15 TRANSFORMED HOST CELLS AND TRANSGENIC PLANTS

Methods and compositions for transforming a bacterium, a yeast or fungal cell, a plant cell, or an entire plant with one or more expression vectors comprising a pheromone-encoding gene segment are further aspects of this disclosure. A

transgenic bacterium, yeast or fungal cell, plant cell, plantlet, or mature plant derived from such a transformation process or the progeny and seeds from such a transgenic plant are also further embodiments of the invention.

Means for transforming bacteria such as *E. coli* or *Salmonella* are well-known to those of skill in the art. Similar means may also be used for transformation of *Agrobacterium*, and in particular *A. tumefaciens* for use in delivery of nucleic acid segments to a plant cell. Means for transforming fungal and yeast cells, such as *M. grisea*, *C. parasitica*, and *S. cerevisiae* are also well-known to those of skill in the art.

U. S. Patents 5,538,880 and 5,538,877 issued to Lundquist and Walters (specifically incorporated herein by reference) disclose microprojectile-based methods for preparing fertile transgenic corn.

U. S. Patent 5,530,193 issued to Clark *et al.* (specifically incorporated herein by reference) discloses a method of producing virus-resistant transgenic corn.

Herbicide-resistant transgenic plants and methods of making are disclosed in U. S. Patent 5,633,435 issued to Barry *et al.* (specifically incorporated herein by reference).

U. S. Patent 5,563,055 issued to Thomas and Townsend (specifically incorporated herein by reference) discloses a method of making transgenic soybeans using *Agrobacterium*-mediated transformation.

Intl. Pat. Appl. Publ. No. WO 9527068 by Beach *et al.* (specifically incorporated herein by reference) discloses methods for making plant seeds which have been genetically modified to express a preselected protein.

The generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Pat. Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference).

Agrobacterium has also been used by Chee *et al.* to successfully transform undifferentiated germinating meristematic or mesocotyl cells (U. S. Patents 5,169,770 and 5,376,543; and WO 8905859-- each of which is specifically incorporated herein by reference).

U. S. Patent 5,597,718 issued to Brill *et al*, U. S. Patent 5,521,078 issued to Maliyakal, and U. S. Patent 5,474,925 issued to Barton and Maliyakal (each of which is specifically incorporated herein by reference) disclose various methods for the

production of transgenic cotton.

Intl. Pat. Appl. Publ. No. WO 9640924 by McBride *et al.* (specifically incorporated herein by reference) describes DNA constructs which are useful in the preparation of transgenic cotton.

Ovary-specific tissue transcription factors have been described for transformation of plants to direct the tissue-specific production of heterologous proteins in transgenic cotton (Intl. Pat. Appl. Publ. No. WO 9626639 by Martineau and Martineau, 1996, specifically incorporated herein by reference).

U. S. Patent 5,349,126 issued to Chappell *et al.* (specifically incorporated herein by reference) describes means for producing transgenic plants such as tomato, alfalfa, barley, carrot, and tobacco, having increased insect resistance.

Fry and Zhou (U. S. Patent 5,631,152, specifically incorporated herein by reference) disclose a rapid transformation regeneration system for obtaining fertile transformed wheat.

Fry and Zhou (Eur. Pat. Appl. Pub. No. EP 709462; 1996, specifically incorporated herein by reference) describe the production of transgenic monocotyledonous plants such as wheat by transforming regenerable tissue or embryogenic calli with a foreign DNA.

U. S. Patent 5612487 issued to Arntzen and Lam (specifically incorporated herein by reference) describes the production of anti-viral transgenic tobacco.

Merikke *et al.* (U. S. Patent 5,589,625, specifically incorporated herein by reference) describes the production of transgenic plants (such as tobacco and potato) which express multiple virus resistance. The method involves the production of transgenic plants comprising recombinant 2.5 alpha synthetase activity.

U. S. Patent 5,422,108 issued to Fitzmaurice and Mirkov (specifically incorporated herein by reference) describes the production of plants (including transgenic tobacco) resistant to bacterial pathogens of the genera *Agrobacterium*, *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Clavibacter*.

Kauppinen *et al.* (Intl. Pat. Appl. Publ. No. WO 9526628, 1995, specifically incorporated herein by reference) disclose a method of generating fertile transgenic barley plants using protoplasts isolated from microspores.

Chang *et al.* (Intl. Pat. Appl. Publ. No. WO 9413822, 1994, specifically

incorporated herein by reference) describe the production of stably transformed fertile wheat plants by bombarding wheat tissue with DNA to develop high-yield, high-nutritional and disease-resistant wheat varieties.

Intl. Pat. Appl. Publ. No. WO 9318168 by Eyal *et al.* (1993, specifically incorporated herein by reference) discloses the production of transgenic wheat containing foreign DNA using aqueous DNA solutions applied to pollinated stigmas of emasculated plant florets, prior to fertilization.

U. S. Patent 5,405,765 (specifically incorporated herein by reference) and Intl. Pat. Appl. Publ. No. WO 9304178 by Vasil and Vasil (1993, specifically incorporated herein by reference) disclose the production of transgenic wheat plants using DNA delivery to type C embryonic callus, to permit expression of cloned genes (*e.g.*, herbicide resistance) in the transformed plant.

Methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is well known which methods are useful for a particular plant strain.

While there are many methods for introducing transforming DNA segments into cells, not all of these have been shown to be suitable for delivering DNA to plant cells. Suitable methods, however, are believed to include virtually any method by which DNA can be introduced into a cell, such as by *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, *etc.* In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, 1973; Zatloukal *et al.*, 1992); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and

Neumann, 1982; Fromm *et al.*, 1985; U. S. Patent No. 5,384,253) and the gene gun (Johnston and Tang, 1994; Fynan *et al.*, 1993); (3) viral vectors (Clapp, 1993; Lu *et al.*, 1993; Eglitis and Anderson, 1988a; 1988b); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992).

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4.15.1 ELECTROPORATION

The application of brief, high-voltage electric pulses to a variety of animal and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of clones genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

The introduction of DNA by means of electroporation, is well-known to those of skill in the art. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation, by mechanical wounding. To effect transformation by electroporation one may employ either friable tissues such as a suspension culture of cells, or embryogenic callus, or alternatively, one may transform immature embryos or other organized tissues directly. One would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Such cells would then be recipient to DNA transfer by electroporation, which may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

4.15.2 MICROPROJECTILE BOMBARDMENT

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles

include those comprised of tungsten, gold, platinum, and the like.

An advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming monocots, is that neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into maize cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus which express the exogenous gene product 48 hrs post-bombardment often range from 1 to 10 and average 1 to 3.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are

especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

4.15.3 *AGROBACTERIUM*-MEDIATED TRANSFER

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described (Fraley *et al.*, 1985; Rogers *et al.*, 1987). Further, the integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, 1986; Jorgensen *et al.*, 1987).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice

because of the facile and defined nature of the gene transfer.

Agrobacterium-mediated transformation of leaf disks and other tissues such as cotyledons and hypocotyls appears to be limited to plants that *Agrobacterium* naturally infects. *Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants. Few monocots appear to be natural hosts for *Agrobacterium*, although transgenic plants have been produced in asparagus using *Agrobacterium* vectors as described (Bytebier *et al.*, 1987). Therefore, commercially important cereal grains such as rice, corn, and wheat must usually be transformed using alternative methods. However, as mentioned above, the transformation of asparagus using *Agrobacterium* can also be achieved (see, for example, Bytebier *et al.*, 1987).

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous gene segregates independently during mitosis and meiosis.

More preferred is a transgenic plant that is homozygous for the added structural gene; *i.e.*, a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced carboxylase activity relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a polypeptide of interest. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

4.15.4 Other Transformation Methods

The formation of transgenic plants may also be accomplished using other methods of cell transformation which are known in the art besides *Agrobacterium*-mediated DNA transfer (Fraley *et al.*, 1983). Alternatively, DNA can be introduced into plants by direct DNA transfer into pollen (Zhou *et al.*, 1983; Hess, 1987; Luo *et al.*, 1988), by injection of the DNA into reproductive organs of a plant (Pena *et al.*, 1987), or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos (Neuhaus *et al.*, 1987; Benbrook *et al.*, 1986).

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, 1988). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene that encodes a polypeptide of interest introduced by *Agrobacterium* from leaf explants can be achieved by methods well known in the art such as described (Horsch *et al.*, 1985). In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described (Fraley *et al.*, 1983).

This procedure typically produces shoots within two to four months and those shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant strain employed, such variations being well known in the art.

Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, as discussed before. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is

used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1988). In addition, "particle gun" or high-velocity microprojectile technology can be utilized (Vasil, 1992).

Using that latter technology, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles as described (Klein *et al.*, 1987; Klein *et al.*, 1988; McCabe *et al.*, 1988). The metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants.

4.16 Methods for Producing Fungus-Resistant Transgenic Plants

By transforming a suitable host cell, such as a plant cell, with one or more recombinant pheromone-encoding gene segments, the expression of the encoded pheromone protein (*i.e.*, a pheromone protein or polypeptide which inhibits fungal growth, appressorium formation, spore germination, conidial development and/or germination) can result in the formation of a fungus-resistant plant.

By way of example, one may utilize an expression vector containing a coding region for a fungal pheromone protein and an appropriate selectable marker to transform a suspension of embryonic plant cells, such as wheat or corn cells using a method such as particle bombardment (Maddock *et al.*, 1991; Vasil *et al.*, 1992) to deliver the DNA coated on microprojectiles into the recipient cells. Transgenic plants

are then regenerated from transformed embryonic calli that express the fungal resistance.

A transgenic plant of this invention comprises one or more fungal pheromone-encoding DNA segments incorporated into its genome, wherein these DNA segments are expressed by the plant to produce the encoded pheromone. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for, by way of example, increased antifungal activity, preferably in the field, under a range of environmental conditions.

The inventors contemplate that the present invention will find particular utility in the creation of transgenic plants of commercial interest including various turf grasses, wheat, corn, cotton, flax, tobacco, hemp, soybeans, rye, rice, barley, oats, a variety of ornamental plants and vegetables, as well as a number of nut- and fruit-bearing trees and plants, as well as shrubs, houseplants, and other plants of commercial interest.

4.17 DNA Sequences as Transgenes

It is well known in the art that virtually any DNA composition may be used for transformation of plant cells to ultimately produce fertile transgenic plants. The construction of vectors which may be employed in conjunction with the present invention will be known to those of skill in the art in light of the present disclosure (see for example, Sambrook *et al.*, 1989; Gelvin *et al.*, 1990). The techniques of the current invention are thus not limited to any particular DNA sequences. For example, DNA segments in the form of vectors and plasmids, or linear DNA fragments, in some instances containing only the DNA element to be expressed in the plant, and the like, may be employed.

In certain embodiments, it is contemplated that one may wish to employ replication-competent viral vectors in plant transformation. Such vectors include, for

example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and PW1-GUS (Ugaki *et al.*, 1991). These vectors are capable of autonomous replication in plant cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector may also be useful for delivery of genes flanked by DNA sequences from transposable elements such as Ac, Ds, or Mu. It has been proposed (Laufs *et al.*, 1990) that transposition of these elements within plant genomes requires DNA replication. It is also contemplated that transposable elements would be useful for introducing DNA fragments lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, *e.g.*, antibiotic resistance genes and origins of DNA replication. It is also proposed that use of a transposable element such as Ac, Ds, or Mu would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells.

It is further contemplated that one may wish to co-transform plants or plant cells with 2 or more vectors. Co-transformation may be achieved using a vector containing the marker and another gene or genes of interest. Alternatively, different vectors, *e.g.*, plasmids, may contain the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. Using this method, the assumption is made that a certain percentage of cells in which the marker has been introduced, have also received the other gene(s) of interest. Thus, not all cells selected by means of the marker, will express the other genes of interest which had been presented to the cells concurrently.

Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) and other DNA segments for use in transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into the cells. These DNA constructs can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells, such as will result in a screenable or selectable trait and/or which will impart an improved phenotype to the regenerated plant. Preferred components likely to be included with vectors used in the current invention are as follows.

4.17.1 Regulatory Elements

Preferred constructs will generally include a plant promoter. Useful promoters include those that are inducible, viral, synthetic, constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989). A promoter is selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Promoters can be near-constitutive, such as the CaMV 35S promoter (Odell *et al.*, 1985), actin, histone, CaMV 19S (Lawton *et al.*, 1987), *nos* (Ebert *et al.*, 1987), Adh (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990), α-tubulin, actin (Wang *et al.*, 1992), cab (Sullivan *et al.*, 1989), PEPCase (Hudspeth & Grula, 1989) and R gene complex-associated promoters (Chandler *et al.*, 1989). Where the promoter is a near-constitutive promoter, increases in polypeptide expression are found in a variety of transformed plant tissues (*e.g.*, callus, leaf, seed and root).

Alternatively, expression of transgenes can be directed to specific plant tissues by using vectors containing a tissue-specific promoter. An exemplary tissue-specific promoter is the lectin promoter (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990.). Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), truncated CaMV 35s (Odell *et al.*, 1985) and Potato patatin promoters (Wenzler *et al.*, 1989). Similarly are the maize zein and globulin-1 promoters. Other tissue specific promoters including root cell promoters (Conkling *et al.*, 1990) and tissue specific enhancers (Fromm *et al.*, 1989) are also contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters.

Another type of element which can regulate gene expression is the DNA sequence between the transcription initiation site and the start of the coding sequence.

termed the untranslated leader sequence. The leader sequence can influence gene expression and compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (Joshi, 1987). Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, will be most preferred.

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers are often found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin gene, and promoters from non-plant eukaryotes (e.g. yeast; Ma *et al.*, 1988).

For monocot transformation, vectors which include the ocs enhancer element are particularly contemplated to be desirable for expression of the fungal pheromone genes of interest. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (ocs) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the ocs element and particularly multiple copies of the element, may be used to increase the level of transcription from adjacent promoters.

It is contemplated that introduction of large DNA sequences comprising more than one gene may be desirable. Introduction of such sequences may be facilitated by use of bacterial or yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes. For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Ultimately, the most desirable DNA segments for introduction into a plant genome may be homologous genes or gene families which encode a desired trait (for

example, resistance to fungal infection), and which are introduced under the control of novel promoters or enhancers, *etc.*, or perhaps even homologous or tissue specific (*e.g.*, root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention may be the production of transformants comprising a transgene which is targeted in a tissue-specific manner. For example, the fungal-resistant genes may be expressed specifically in tissues such as whorl, collar/sheath, and/or root tissues.

Vectors for use in tissue-specific targeting of gene expression in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure.

It is also contemplated that tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for a fungal pheromone protein may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Alternatively, a rice actin promoter or a histone promoter from a dicot or monocot species could also be used for constitutive expression of a gene. Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, U. S. Patent 5,491,288 (specifically incorporated herein by reference) discloses combining a Cauliflower Mosaic Virus promoter with a histone promoter. Thus, expression of an antisense transcript of the pheromone gene in a grain kernel, using for example a zein promoter, would prevent accumulation of the pheromone protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel.

Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which

gene product is relatively abundant in specific tissues. The promoter and control elements of corresponding genomic clones may then be localized using the techniques of molecular biology known to those of skill in the art.

It is proposed that in some embodiments of the present invention expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing is frequently correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated in the endosperm about 15 days after pollination.

It is also contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful to target a gene in order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation replace an existing gene in the cell.

4.17.2 TERMINATORS

The inventors contemplate that in most circumstances genetic constructs comprising the pheromone-encoding genes will typically further comprise a 3' DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as Adh intron 1 (Callis *et al.*, 1987), sucrose synthase intron (Vasil *et al.*, 1989) or TMV omega element (Gallie, *et al.*, 1989), may further be included where desired.

4.17.3 TRANSIT OR SIGNAL PEPTIDES

Sequences that are joined to the coding sequence of the fungal resistance gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes are termed transit (usually into vacuoles, vesicles, plastids and other

intracellular organelles) and signal sequences (usually to the outside of the cellular membrane). By facilitating the transport of the pheromone protein into compartments inside and outside the cell these sequences may increase the accumulation of antifungal gene product, thus protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It is further contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (Intl. Pat. Appl. Publ. No. WO 95/24492, specifically incorporated herein by reference).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, *e.g.*, vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

A particular example of such a use concerns the direction of a fungal resistance gene, such as the fungal pheromone genes disclosed herein, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the *rbcS* transit peptide, the chloroplast transit peptide described in Eur. Pat. Appl. Publ. No. EP 0189707 (specifically incorporated herein by reference), or the optimized transit peptide described in U. S. Patent 5,510,471 (specifically incorporated herein by reference), which confers plastid-specific targeting of proteins. In addition, it is proposed that it may be desirable to target certain genes responsible

for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole.

4.17.4 MARKER GENES

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, *i.e.*, by "screening" (*e.g.*, the R-locus trait). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*, α-amylase, β-lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the

cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of maize HPRG (Steifel *et al.*, 1990) is preferred, as this molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller *et al.*, 1989) could be modified by the addition of an antigenic site to create a screenable marker.

4.17.5 Selectable Markers

Many selectable marker genes are described and known in the art which may be used in connection with the present invention including. Such genes include, but are not limited to, a *neo* gene (Potrykus *et al.*, 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; a *bar* gene which confers bialaphos resistance; a mutant EPSP synthase protein (Hinchee *et al.*, 1988) conferring glyphosate resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (Eur. Pat. Appl. Publ. No EP 154204, specifically incorporated herein by reference); a methotrexate resistant DHFR gene (Thillet *et al.*, 1988), a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (Eur. Pat. Appl. Publ. No. EP 0218571, specifically incorporated herein by reference).

4.17.6 Screenable Markers

In addition to selectable markers, various screenable marker genes may be employed for plant transformation. Objectives which may be achieved using screenable markers include: (1) the detection of expressing colonies in a population, which may not necessarily employ a visible marker; (2) the visualization, by

microscope or unaided eye, of expressing cells within a population or tissue; and (3) the ability to assess tissue- and/or cell-specific expression in gene expression studies. A screenable marker which is capable of being used to meet one of these objectives would be useful and one that meets more than one criteria would be particularly advantageous.

Screenable markers that may be employed include a β-glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a β-lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a *xylE* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β-galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, 1995).

A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein is contemplated as a particularly useful reporter gene (Sheen *et al.*, 1995). Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light.

4.18 Construction of Transgenes for Producing Fungi-Resistant Plants

A particularly important part of the present invention is that it provides methods and compositions for the efficient transformation of plant cells with genes in addition to, or other than, marker genes. Such transgenes will often be genes that direct the expression of a particular protein or polypeptide product, but they may also be non-expressible DNA segments, *e.g.*, transposons such as Ds that do not direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (*e.g.*, mRNA, antisense RNA, *etc.*) or translated into a protein, expressed as a trait of interest, or the like, *etc.*, and is not limited to selectable, screenable or non-selectable marker genes. The invention also contemplates that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

In preferred embodiments, fungal pheromone genes which confer fungal resistance or tolerance to a plant transformed with such genes are particularly preferred. In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more pheromone transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids bearing the *M. grisea* and *S. cerevisiae* mating-type specific pheromone expression units in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of a fungal resistance gene, such as a mating type specific pheromone gene, along with a protease inhibitor gene such as *pinII*.

Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of

interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited above are provided only by way of example and do not represent the full range of applications.

Genes may also be constructed or isolated, which when transcribed produce RNA enzymes, or ribozymes, which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNAs can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including, but not limited to, the polypeptides cited above that may be affected by antisense RNA.

It is also possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by a mechanism of cosuppression. It has been demonstrated in tobacco, tomato, and petunia (Goring *et al.*, 1991; Smith *et al.*, 1990; Napoli *et al.*, 1990; van der Krol *et al.*, 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

4.19 Gene Expression

While Southern blotting and PCR™ may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use

of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and ^{14}C-acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including, but not limited, to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

4.20 PURIFICATION OF PROTEINS

It may, in particular embodiments of the current invention, be desirable to purify proteins encoded by transgenes of the current invention. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific

activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low-pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High performance liquid chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a

very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor-determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and Helix pomatia lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been

used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

4.21 Generating Antibodies Reactive With Transgene Proteins

In another aspect, the present invention contemplates an antibody that is immunoreactive with a transgene molecule of the present invention, or any portion thereof. An antibody can be a polyclonal or a monoclonal antibody. In a preferred embodiment, the antibody is a monoclonal antibody. Means for preparing and characterizing antibodies are well known in the art (see, e.g., Howell and Lane, 1988).

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Antibodies, both polyclonal and monoclonal, specific for isoforms of antigen may be prepared using conventional immunization techniques, as will be generally known to those of skill in the art. A composition containing antigenic epitopes of the compounds of the present invention can be used to immunize one or more

experimental animals, such as a rabbit or mouse, which will then proceed to produce specific antibodies against the compounds of the present invention. Polyclonal antisera may be obtained, after allowing time for antibody generation, simply by bleeding the animal and preparing serum samples from the whole blood.

It is proposed that the monoclonal antibodies of the present invention will find useful application in standard immunochemical procedures, such as ELISA and Western blot methods and in immunohistochemical procedures such as tissue staining, as well as in other procedures which may utilize antibodies specific to transgene-related antigen epitopes. Additionally, it is proposed that monoclonal antibodies specific to a particular transgene protein of different species may be utilized in other useful applications.

In general, both polyclonal and monoclonal antibodies against transgene-encoded proteins may be used in a variety of embodiments. For example, they may be employed in antibody cloning protocols to obtain cDNAs or genes encoding other, related proteins. They may also be used in inhibition studies to analyze the effects of transgene-related peptides in plant cells, plant parts, or whole plants. Anti-transgene encoded protein antibodies will also be useful in immunolocalization studies to analyze the distribution of encoded protein during various cellular events or stages of development. A particularly useful application of such antibodies is in purifying native or recombinant polypeptides, for example, using an antibody affinity column. The operation of all such immunological techniques will be known to those of skill in the art in light of the present disclosure.

Means for preparing and characterizing antibodies are well known in the art (see, *e.g.*, Harlow and Lane, 1988; incorporated herein by reference). More specific examples of monoclonal antibody preparation are given in the examples below.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde,

m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As also is well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster, injection may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate mAbs.

mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U. S. Patent 4,196,265 (specifically incorporated herein by reference). Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified transgene-encoded protein, polypeptide or peptide or cell expressing high levels of the transgene. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B-lymphocytes (B-cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or

lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, 1986; Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, P3-X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 ratio, though the ratio may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described (Kohler and Milstein, 1975; 1976), and those using polyethylene glycol (PEG), such as 37% (vol./vol.) PEG, by Gefter *et al.*, (1977). The use of electrically induced fusion methods is also appropriate (Goding, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, around 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused

myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B-cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B-cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide mAbs. The cell lines may be exploited for mAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide mAbs in high concentration. The individual cell lines could also be cultured *in vitro*.

where the mAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. mAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

5

4.22 IMMUNOASSAYS FOR TRANSGENE EXPRESSION

Antibodies of the present invention can be used in characterizing the expression of transgenes used in the current invention, through techniques such as ELISAs and Western blotting. This may provide a more efficient, accurate or cost 10 effective method to screen transgenic plants than other assays.

The use of antibodies of the present invention, in an ELISA assay is contemplated. For example, anti-transgene-encoded proteins are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed 15 material, it is desirable to bind or coat the assay plate wells with a non-specific protein that is known to be antigenically neutral with regard to the test antisera such as bovine serum albumin (BSA), casein or solutions of powdered milk. This allows for blocking of non-specific adsorption sites on the immobilizing surface and thus reduces the background caused by non-specific binding of antigen onto the surface.

20 After binding of antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the sample to be tested in a manner conducive to immune complex (antigen/antibody) formation.

Following formation of specific immunocomplexes between the test sample 25 and the bound antibody, and subsequent washing, the occurrence and even amount of immunocomplex formation may be determined by subjecting same to a second antibody having specificity for proteins that differ from the first antibody. Appropriate conditions preferably include diluting the sample with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween®. 30 These added agents also tend to assist in the reduction of nonspecific background. The layered antisera is then allowed to incubate for from about 2 to about 4 hr, at temperatures preferably on the order of about 25° to about 27°C. Following

incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween®, or borate buffer.

To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (*e.g.*, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween®).

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline)-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectrum spectrophotometer.

The preceding format may be altered by first binding the sample to the assay plate. Then, primary antibody is incubated with the assay plate, followed by detecting of bound primary antibody using a labeled second antibody with specificity for the primary antibody.

4.23 ANTISENSE CONSTRUCTS

Antisense treatments are one way of inhibiting the expression of a particular gene in a plant. Antisense technology may be used to "knock-out" the function of a particular endogenous or exogenous gene, thereby decreasing or eliminating the expression of the targeted gene in a transformed plant cell or whole plant.

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired

with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would

bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

Contemplated as being particularly useful will be antisense constructs which are expressed in a tissue-specific manner. By regulating expression of the antisense construct with a tissue-specific promoter, one may eliminate the expression of a gene or transgene in a particular tissue. For instance, one may wish to eliminate expression of an insect toxin gene from the kernel. This would simply be achieved by the kernel specific expression of an antisense construct for the insect resistance gene. This would allow, for example, the use of a constitutive promoter for the gene to be expressed, but by tissue specific expression of antisense DNA, a tissue specific pattern of expression is achieved. This may be done to regulate the expression of a particular transgene or gene in potentially any tissue. A similar strategy may also be employed to achieve other types of regulation of gene expression, such as developmental or temporal regulation.

4.24 DNA PREPARATION

In some instances it will be desirable to deliver DNA to plant cells that does not contain DNA sequences necessary for maintenance of the plasmid vector in the bacterial host, *e.g.*, *E. coli*, such as antibiotic resistance genes, including but not limited to ampicillin, kanamycin, and tetracycline resistance, and prokaryotic origins of DNA replication. In such case, a DNA fragment containing the transforming DNA may be purified prior to transformation. An exemplary method of purification is gel electrophoresis on a 1.2% low melting temperature agarose gel, followed by recovery from the agarose gel by melting gel slices in a 6-10 fold excess of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 70°C-72°C); frozen and thawed (37°C); and the agarose pelleted by centrifugation. A Qiagen Q-100 column may then be used for purification of DNA. For efficient recovery of DNA, the flow rate of the column may

be adjusted to 40 ml/hr.

Isolated DNA fragments can be recovered from agarose gels using a variety of electroelution techniques, enzyme digestion of the agarose, or binding of DNA to glass beads (*e.g.*, Gene Clean). In addition, HPLC and/or use of magnetic particles may be used to isolate DNA fragments. As an alternative to isolation of DNA fragments a plasmid vector can be digested with a restriction enzyme and this DNA delivered to maize cells without prior purification of the expression cassette fragment.

4.25 IDENTIFICATION OF TRANSFORMED CELLS USING SELECTION

Because artisans in the field of plant transformation methodologies generally believe that DNA is introduced into only a small percentage of target cells in any one study, in order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes it is often desirable to use selection means for obtaining those cells that have been stably transformed by the exogenous DNA. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphostransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells are then exposed to the selective agent. In the population of surviving cells will be those cells wherein generally the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Using the techniques disclosed herein, greater than 40% of bombarded embryos may yield transformants.

One herbicide which has been suggested as a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine

residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ is also effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in German Patent DE 3642829A (specifically incorporated herein by reference), wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block, 1987) and *Brassica* (De Block, 1989) plants.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U. S. Patent 4,535,060 (specifically incorporated herein by reference) describes the isolation of EPSPS mutations which infer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate-resistant *aroA* gene were introduced *in vitro*.

Mutant genes encoding glyphosate-resistant EPSPS enzymes are described in, for example, Intl. Pat. Appl. Publ. No. WO 97/4103 (specifically incorporated herein by reference). The best-characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful.

It is further contemplated that the herbicide DALAPON, 2,2-dichloropropionic

acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U. S. Patent 5,508,468 (specifically incorporated herein by reference).

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 -- FUNGAL PHEROMONES BLOCK INFECTION AND PATHOGENICITY OF RICE BLAST FUNGUS

Germlings of the rice blast fungus, *M. grisea*, form an infective structure called an appressorium that is necessary for pathogenesis. α-Factor pheromone of the yeast *S. cerevisiae* inhibited appressorium formation of *M. grisea* in a mating type-specific manner. An extract from *M. grisea* mating type-*MAT1-1* culture filtrates inhibited appressorium formation in *MAT1-2* but not *MAT1-1* isolates. This example demonstrates that yeast extract inhibits appressorium formation of two *M. grisea* strains: the barley pathogen 4091-5-8, and the rice pathogen, Guy11. The response of a number of *M. grisea* strains was tested and it was found that all mating type *MAT1-2* strains (such as 4091-5-8 and Guy11) were strongly inhibited for appressorium formation by yeast extract (Table 2, FIG. 4A, FIG. 4B). Surprisingly, none of the *MAT1-1* strains showed such a strong inhibition (Table 2, FIG. 4E). Progeny from a cross of strain CP987 (*MAT1-1*) with strain 4091-5-8 (*MAT1-2*) were tested and it was found that 16 of the 31 progeny were sensitive to yeast extract. The mating type of each of these strains was determined by crosses to both parents. The 16 progeny

sensitive to the appressorium inhibiting activity of yeast extract were *MAT1-2*, while the resistant strains were *MAT1-1*. The absolute correlation of the inhibition of appressorium formation with mating type suggested a pheromone-like response to yeast extract.

Yeast extract (Difco Laboratories, Detroit MI Lot #8549JE) was subjected to a benzyl alcohol extraction protocol designed to isolate hydrophobic peptides (Garibaldi and Neilands, 1955). The benzyl alcohol fractionation of yeast extract was prepared by a modification of the method of Garibaldi and Neilands (1955). The yeast extract solution was titrated to pH 3.0 with dilute sulfuric acid, saturated with ammonium sulfate (70.6 g/0.1 L) and extracted with benzyl alcohol three times. The benzyl alcohol fractions were pooled and extracted with diethyl ether. This benzyl alcohol fraction was extracted with water three times, back extracted with ether, and the aqueous phase was lyophilized. The residue was dissolved in water, filter sterilized and stored at -20°C. Extraction of putative pheromone-like activity of *M. grisea* was performed as above with the following exceptions: *M. grisea* strains 987 and 4091 were grown on 100 ml oatmeal broth (OMB 50 g oatmeal per L), separately and co-inoculated culture). Media was drained and extracted as above. Uninoculated OMB was utilized as a control. This extract retained the mating type-specific activity (Table 2). The activity in the benzyl alcohol extract could be resolved by thin-layer chromatography using solvent conditions designed for purification of yeast α-factor pheromone (Duntze *et al.*, 1973). Thin-layer chromatography was performed as described. The benzyl alcohol extracted yeast extract was spotted onto a silica gel fluorescent TLC plate and resolved with *n*-butanol-acetic acid-water. The chromatograms were viewed under UV fluorescence and stained with 1% ninhydrin in acetone. The fractions were extracted from parallel lanes by scraping sections of the silica gel into a microfuge tube and extracting with acidified ethanol. The active fraction had an R_f of approximately 0.35.

The preceding findings caused the inventors to consider whether the yeast extract contained a fungal pheromone mimic, or even authentic yeast pheromone. Structural and functional similarities of peptide hormones have been recognized previously. α-Factor pheromone has activity against other yeast species (Hisatomi *et al.*, 1988), however, the ability of α-factor to function in a pheromone-like manner in

such a distant relative as *M. grisea* has not been demonstrated previously. Remarkably, yeast α-factor pheromone has sequence similarity to mammalian gonadotropin-releasing hormone and stimulates the release of luteinizing hormone from cultured gonadotrophs, although it was 9000-fold less active (Loumaye *et al.*, 1982). An evolutionary conservation of hormones involved in sexual reproduction was suggested (Loumaye *et al.*, 1982).

The activity of synthetic *S. cerevisiae* α-factor pheromone (Sigma, St. Louis, MO) was tested and found to be similar to that of yeast extract: appressorium development was inhibited in *MAT1-2* strains (Table 2, FIG. 4C). However, *MAT1-1* strains were completely unaffected by α-factor and produced appressoria at high frequency (Table 2, FIG. 4F). The concentration of α-factor needed to inhibit appressorium formation of *MAT1-2* strains by greater than 95% was 0.5 mg/ml (3×10^{-4} M). This is roughly 10^5 fold higher than the concentration of α-factor required to observe a biological effect with *S. cerevisiae* cells (Raths *et al.*, 1988), but similar to the levels found to have activity in cultured gonadotrophs (Loumaye *et al.*, 1982).

For appressorium formation assays with a-factor, conidia were quantitated, and 10^4 conidia/ml were used for the assays. Yeast a-factor was dissolved in DMSO to a final concentration of 1 mg/ml. One, five and ten μl were spotted onto the hydrophobic side of gelbond and allowed to dry for 24 h prior to assaying. Conidia were placed on top of the dried residue and were incubated as previously described. The yeast shmoo morphology assay was performed in microtiter dish wells. Strains W303a and W303a were challenged with α-factor (Sigma) and a-factor pheromone controls. The benzyl alcohol fraction of yeast extract was tested similarly.

It was found that the a-factor pheromone of *S. cerevisiae* did not inhibit appressorium formation of either mating type of *M. grisea* (Raths *et al.*, 1988). The solubility of the lipophilic a-factor may be too low to allow stimulation of an a-factor receptor homolog.

The amount of yeast extract needed to inhibit appressorium formation was approximately 20 mg/ml. This suggests that either (i) α-factor is present as roughly 2.5% of the dry weight of yeast extract; (ii) α-factor is present in low concentrations and its activity is stimulated by other factors in yeast extract; or (ii) yeast extract serendipitously contains another activity that can inhibit appressorium formation in a

mating type-specific manner in *M. grisea*. It is considered unlikely that yeast extract contains sufficient levels of α-factor to block appressorium formation. The inventors were unable to detect α-factor activity against *S. cerevisiae* using yeast extract or the benzyl alcohol fraction obtained from yeast extract (Raths *et al.*, 1988).

Regardless of the nature of the activity in yeast extract it appears that *M. grisea* possesses pheromone receptors and corresponding pheromones with similarity to those of *S. cerevisiae*. To determine whether an activity from *M. grisea* could be identified that possessed appressorium-inhibiting activity benzyl alcohol extracts were prepared from culture filtrates of both mating types grown on a medium conducive to mating (Garibaldi and Neilands, 1955). An extract from uninoculated broth had no effect on growth or development. However, the benzyl alcohol fraction from strain CP987 (*MAT1-1*) inhibited appressorium formation of strain 4091-5-8 (*MAT1-2*) but did not affect germinated conidia of strain 987 (FIG. 4D and FIG. 4G). Thus, the extract from strain CP987 contains an activity with a specificity similar to α-factor. The benzyl alcohol fraction from strain 4091-5-8 did not inhibit appressorium formation of either strain. An a-factor like isoprenylated peptide would be extremely hydrophobic and would not likely be obtained efficiently by the extraction scheme employed here (Garibaldi and Neilands, 1955). The simplest model to explain the findings is that yeast α-factor interacts weakly with a corresponding pheromone receptor of *M. grisea*. It is also possible that the mating type-specific activity from *M. grisea MAT1-1* is a pheromone with similarity to yeast α-factor.

One corollary to the model presented above is that pheromone receptors of *M. grisea* and other fungi may be useful as targets for plant protection by pheromones of pheromone derivatives. To test the usefulness of this concept and to examine whether the effects observed *in vitro* could be replicated in *planta*, barley plants were inoculated with *M. grisea* conidia of strain 4091-5-8 in the presence of yeast α-factor (FIG. 5A, FIG. 5B, FIG. 5C and FIG. 5D). A significant reduction in lesion density and diseased leaf area was observed in two independent studies: Mean lesion densities for 12.5 cm of leaves were 35.5 lesions for the control versus 3.1 lesions for the pheromone treated plants ($t = -6.506$, $P < 0.0001$, $df = 13$). In one study, a set of plants was sprayed with conidia and a 22 residue peptide with no sequence similarity to α-factor. No protection of plants by this control peptide was observed (FIG. 5A,

FIG. 5B, FIG. 5C, and FIG. 5D).

In several pathogenic fungi, a relationship between morphology, the mating response pathway, and virulence is beginning to be elucidated. In *Ustilago maydis*, two unlinked loci regulate morphology, mating and pathogenicity (Bölker *et al.*, 1992; Spellig *et al.*, 1994). Studies with adenylate cyclase and cAMP-dependent protein kinase regulatory subunit mutants suggest an interaction between the mating pathway and a cAMP-dependent pathway in controlling the switch to the filamentous growth required for pathogenesis (Gold *et al.*, 1994). The *CPH1* gene from *Candida albicans*, appears to be homologous to the *S. cerevisiae* gene *STE12* (Liu *et al.*, 1994). The diploid *cph1* mutant is suppressed for filamentous growth on solid media. This is an important phenotype because filamentous growth is associated with virulence. *STE12* is a component of both the pheromone response and the invasive response pathways in *S. cerevisiae*. The invasive response shares many components required for pseudohyphal growth in diploids, including *STE12* (Herskowitz, 1995). Pseudohyphal growth is stimulated by constitutive activation of the RAS2/cAMP pathway (Gimeno *et al.*, 1992).

In *M. grisea*, the role of cAMP in pathogenic development has been demonstrated by induction of appressorium formation by exogenous cAMP and the loss of development caused by mutation of the cAMP-dependent protein kinase (Lee and Dean, 1993; Mitchell and Dan, 1995). Furthermore, induction of appressorium formation in germlings by cAMP was shown to occur only during a specific stage of growth. This indicates that recognition of a potential host plan triggers an increase in cAMP levels to initiate development (Lee and Dean, 1993). The finding that cAMP can override the effect of α-factor (FIG. 4H) suggests that one result of pheromone receptor stimulation in germlings is to block this increase in cAMP levels. Studies of the effect of pheromone on cAMP levels in *S. cerevisiae* suggest a similar phenomenon. Although α-factor does not appear to affect basal adenylate cyclase activity (Casperson *et al.*, 1983), stimulation of α-factor receptor blocks the increase in cAMP levels induced by glucose addition to cells (Papasavvas *et al.*, 1992).

Yeast α-factor and the other pheromone-like activities examined did not inhibit germination or growth of *M. grisea*. Furthermore, when conidia of opposite mating types were mixed a reduction of appressorium formation was not observed.

For the germling co-inoculation assay conidia of 4091-5-8, 4360-R-12, CP987 and 4360-R-17 were used. Conidia were quantitated and diluted to 10^4 conidia per ml and mixed in all possible pairwise combinations. Germlings were inoculated onto the hydrophobic side of GelBond film (FMC) and incubated overnight prior to scoring appressorium formation. It is possible that germlings possess pheromone receptors but that they do not produce enough pheromone-like activity to block appressorium formation. One possible scenario in nature is that a germling may attempt to infect the host unless it is near to an established colony of the opposite mating type. Appressorium formation would be blocked by pheromone produced by the colony and the germling would continue to grow and subsequently mate. Interestingly, natural isolates of *M. grisea* that infect rice are usually found to be infertile, hence, it will be of interest to determine whether α-factor and the pheromone-like activities described here will have activity against typical field isolates of the rice blast fungus.

Presumably, the authentic mating factors from *M. grisea* will have biological activity at a concentration similar to that required to induce the shmoo morphology in *S. cerevisiae* (approximately 10^{-9} M) (Raths *et al.*, 1988). The ease of the appressorium formation bioassay suggests the potential for rapid development of novel plant protection agents. Whether the activity from *M. grisea* is shown to be a mating pheromone or some other compound, the pheromone receptor is the likely target. For example, novobiocin has been found to stimulate the α-factor receptor of *S. cerevisiae* and inhibit growth in a mating type specific manner (Pocklington and Orr, 1994).

TABLE 2

YEAST EXTRACT AND YEAST ALPHA-FACTOR INHIBIT APPRESSORIUM FORMATION

Strain[a]	Mating type	buffer	2% YE	Benzyl-alcohol fraction	0.5 mg/ml α-factor
Guy11	1-2	92	0.0	2.2	0.8
4091	1-2	95	0.0	0.7	0.5
4360-12	1-2	92	0.0	2.2	0.4
4375-R-6	1-2	97	0.0	1.0	0.0
CP987	1-1	98	43	56	91
4360-R-17	1-1	96	26	44	94
4136-4-3	1-1	92	50	51	86
4375-R-26	1-1	88	47	44	91

[a]Assays were performed as described in 10 µl assays. Conidia were resuspended in 10 mM phosphate buffer, pH 6.5, 2% yeast extract (YE), a benzyl alcohol extracted fraction of yeast extract, and 0.5 mg/ml yeast α-factor pheromone. Values shown are the percentage of conidia that formed at least one appressorium.

5.2 EXAMPLE 2 -- CHARACTERIZATION OF *C. PARASITICA* PHEROMONE BINDING

It was important, however, to determine the biological manifestations of disruption of pheromone binding in the filamentous ascomycetes. Such studies have been performed in *C. parasitica*. This ascomycete is a good model system for studies of filamentous plant pathogens. Transformation efficiency is among the highest reported for filamentous fungi (10^5 transformations per µg plasmid DNA) (Churchill *et al.*, 1990). The inventors have used homologous recombination methods to delete specific genes (Zhang *et al.*, 1993; Kim *et al.*, 1995) and have cloned genes using marker rescue and by complementation of mutants. Sexual crosses are easily done in the laboratory (Anagnostakis, 1979).

Initial studies have suggested that pheromones may play a role in asexual sporulation. This observation was based upon creation of a null mutation of a pheromone precursor gene. This example describes the preparation and cloning of null mutants of a number of different genes that are likely to be involved in the pheromone response pathway of *C. parasitica*. These genes include the already cloned pheromone precursor genes, the pheromone receptors, and a key component of the MAPK signal transduction pathway. One such example is the homolog of *Ste7*, a component of the yeast MAPK pheromones response pathway. Disruption of this gene in yeast and *U. maydis* (Banuett and Herskowitz, 1994) block the pheromone receptor, as well as other phenotypes, in both of these fungi. From what is known of

pheromone receptors in *S. cerevisiae* and *S. pombe*, disruption of the pheromone precursor genes, the receptor genes, and a component of the putative signal transduction pathway provide a good understanding of the biological response of *C. parasitica* to pheromone binding.

5.3 EXAMPLE 3 -- CLONING OF PHEROMONE RECEPTOR GENES

This example describes the isolation, characterization and cloning of the pheromone receptor genes of *C. parasitica*.

It can be assumed that the pheromone receptors of *C. parasitica* are members of the β-adrenergic receptor superfamily of receptors, as are those of the yeasts (Strader *et al.*, 1994). A large number of members of this class of receptors have been sequenced, so much is known about conserved regions that could be used to amplify sequences using PCR™. Among the yeasts, there is high sequence similarity in the loop region between the first two membrane spanning regions. This sequence appears to be conserved in a wide range of these receptors (Marsh and Herskowitz, 1988), including *U. maydis* (Bolker *et al.*, 1992). In addition, there are a number of other conserved regions in these genes that offered suitable PCR™ primers. Using primers based upon the sequences from *S. cerevisiae*, there is likely enough similarity between the filamentous ascomycetes and the ascomycetous yeasts for primers of conserved regions to successfully clone these receptors from filamentous fungi. Design of primers will likely be most successful if sequences for the receptors for prenylated pheromones and those for non-prenylated pheromones are separately considered since the similarity between these two receptors of *S. cerevisiae* is less than between similar receptor types of different fungi.

Although the pheromone mRNAs are relatively abundant transcripts (Kazmierczak *et al.*, 1996) it cannot be assumed that the same will be the case with receptor mRNAs. Unlike the yeasts, the filamentous ascomycetes may only express pheromone receptors in the trichogyne and ascogonium. To increase the copy number of receptor transcripts in these tissues, the inventors pretreat cultures with pheromones of the opposite mating type. This is expected to provide cloning of these genes as cDNAs. Since there are probably copies of both receptors in both mating types of the fungus, just as there are silent copies of the pheromone precursor genes, it would not

be easy to clone these genes using a method to detect small differences in genomic DNA, such as that of Lisitsyn *et al.* (1993). If PCR™ amplification of cDNAs is not successful, primers may be chosen for genomic rather than cDNA amplification.

Another approach for cloning of the receptors involves direct cloning of a cDNA library into yeast. Basically, a *C. parasitica* library is transformed into yeast and clones containing receptor genes identified by the response of yeast to pheromone binding.

The homolog of *Ste7* of *S. cerevisiae* (FIG. 3) has recently been cloned in the inventors' laboratory. The cloning was accomplished by design of PCR™ primers from conserved sequences of the gene from different organisms. Searches of databases have shown that the best match of the sequence is with the comparable gene of *S. pombe*.

5.4 EXAMPLE 4 -- DELETION MUTATION OF CLONED GENES

This example describes the construction of deletion mutants of various fungal genes. Each of the pheromone precursor genes, the pheromone receptor genes, and the *Ste7* homolog are individually deleted from *C. parasitica*. The inventors have successfully deleted a number of genes from this fungus, including *Vir2*, one of the pheromone precursor genes, so the inventors do not expect a problem with this procedure. The basic approach is as described in Zhang *et al.* (1993) in which the coding region of the gene to be deleted is replaced in a vector with an antibiotic selectable marker, such as the *HygB* gene. At least 1 kb of sequence flanking both sides of the gene is included in the vector. A double mutant strain is created from which both *Vir1* and *Vir2* are deleted. Starting with the strain from which *Vir2* has been deleted, *Vir1* is then deleted as described above, except that the vector is constructed using a benomyl resistance gene as the selectable marker since the *Vir2* deletion strain already contains the *hygB* gene.

Although these deletions will cause sterility, they are not lethal. This limits the ability to demonstrate, through a sexual cross, that the null mutation causes the observed phenotype. The inventors have investigated whether or not deletion causes the observed phenotype by using complementation analysis of the mutation with a wild-type copy of the gene. In the case of *Vir2*, this strategy resulted in a restoration

of asexual sporulation to normal levels, but not fertility (Zhang *et al.*, 1993).

5.5 Example 5 -- Bioassays To Measure Pheromone Binding In Ascomycetes

This example describes the development of a means to identify antagonists of pheromone binding that could be useful for the control of a number of different plant diseases. The problem has been that most of the fungi of interest are not easily manipulated using molecular genetic tools. Also, bioassays that measure the response of trichogynes to applied pheromones, such as used with *Neurospora* (Islam, 1981), have been too cumbersome and slow to be useful in screening potential antagonists of binding. Since the pheromones are quite specific in their binding (Marsh and Herskowitz, 1988), antagonists that are identified are expected to be receptor specific and unlikely to inhibit binding to other receptors. A novel assay has been developed that uses receptors from a number of different fungi.

Previous work has shown that the receptor for the α pheromone of *S. kluyveri* will function in *S. cerevisiae* (Marsh and Herskowitz, 1988) and initiate the pheromone response pathway when treated with the *S. kluyveri* α-pheromone. If the pheromone receptors of other ascomycetes can also function in yeast, the well-developed yeast pheromone-response bioassays can be used to identify antagonists to the receptor. The plate growth inhibition assay is clear enough that it may be used to identify anonymous clones containing pheromone receptor genes from other fungi. With this type of assay it is not necessary to measure trichogyne growth as an assay for pheromones.

This assay requires a functional interaction between the foreign receptor protein and the resident Gα subunit of *S. cerevisiae*. Without functional interaction, a Gα subunit from *C. parasitica* or *N. crassa* cannot be coexpressed in a yeast strain deficient for the resident Gα subunit (*gpa1*), as reported by King *et al.* (1990) for the successful expression of a mammalian β-adrenergic receptor in yeast.

The mammalian receptor was able to induce the yeast pheromone response pathway. Although the yeast cells expressing the mammalian receptor were unable to mate, the *Fus1* gene was stimulated by the mammalian receptor to the same extent as in normal cells induced by yeast pheromone. Fusion of the *Fus1* gene to *LacZ* provided a clear and quantitative assay for receptor binding.

In those instances where the pheromone receptors of a filamentous fungi function directly in yeast, a fungal Gα subunit clone is used as described by King *et al.* (1990). The recent cloning and sequencing of two Gα subunits from *N. crassa* (Turner and Borkovich, 1993) and two from *C. parasitica* (Choi *et al.*, 1995) have made such clones available for vector construction by PCR™ amplification of the Gα subunit cDNAs. It is not known if any of the Gα subunits cloned from filamentous fungi normally function in pheromone response pathways. Sequences of Gα subunits are highly conserved so it may be possible to identify which filamentous fungal Gα subunit will function in the yeast pheromone-response pathway by complementing a yeast *gpa1* (Gα subunit of pheromone response pathway) mutation with clones of the various Gα subunit genes of filamentous fungi.

As required, the various fungal Gα subunit genes are cloned into a yeast vector as described by King *et al.* (1990). For strains in which none of the known Gα subunits from filamentous fungi function in the pheromone response pathway, anonymous cDNA clones of *C. parasitica* have been screened in yeast for complementation of *gpa1*. A cosmid library of *C. parasitica* was constructed from which genes were cloned by complementation at a frequency of about 1 in 5000 transformants. A cDNA library requires screening many more colonies, but the ease of working with yeast, and the simple plate assay for expression of a complementing clone, more than compensate for having to use only cDNAs for complementation studies.

One of the potential advantages of this bioassay is its exploitation as a tool for cloning receptor genes from various fungi. The basic strategy is as described above, *i.e.*, yeast strains lacking the respective receptors are transformed with libraries of different fungi. After plating, the cells are treated with pheromones. Clones containing pheromone receptor genes that are able to complement the yeast mutation are identified by either growth inhibition (G_1 arrest) or expression of a *Fus1:LacZ* fusion protein. In addition to this approach of cloning receptor genes from various fungi, the same primers would likely function in cloning the pheromone receptors from these other ascomycetes.

For cloning of receptor genes, or study of pheromone reception, active pheromone peptides are needed. In the case of *S. cerevisiae*, each of the pheromone

peptides have been synthesized and found to be as active as the native pheromones (Xue *et al.*, 1989). It is assumed that similarly, synthetic peptide pheromones of *C. parasitica* and other filamentous ascomycetes will be active in bioassays. The decapeptide pheromone of *C. parasitica* mating type a may be obtained commercially, and the farnesylated pheromone may be synthesized commercially as well (Quality Controlled Biochemicals, Inc., Hopkinton, MA). Synthetic *C. parasitica* pheromones may be synthesized based upon processing sites of the yeast pheromones. For cases where synthetic pheromones are inactive, the inventors have tested step-wise elimination of the N-terminal amino acids has in some instances produced an active peptide.

The relative concentration of pheromone peptides that inhibit the germination of conidia has been measured. The peptide synthesized following the Matl pheromone sequence totally inhibits germination of conidia of a Mat2 strain at a concentration of 10^{9} M (2.5 ng/ml). There is an approximate 50% inhibition at 10^{-10} M.

Furthermore, inhibition of germination of the conidia of the Matl mating type also occurred at concentrations of 10^{-6} M and higher, and inhibition of germination of conidia of both mating types appeared to be irreversible, unlike the situation with G1 arrest of yeast.

Enzymes necessary to process and secrete peptides similar to the Matl pheromone of *Cryphonectria parasitica* have been detected in plants. The secretion of the Matl pheromone of *C. parasitica* involves processing by a Kex2p system of enzymes. A heterologous protein that requires Kex2p processing was found to be successfully secreted by a plant (Kinal *et al.*, 1995). The implication is that the fungal pheromone precursor genes can be transformed into plants, expressed and secreted. Thus, secretion of the pheromone should protect plants from infection by specific fungal pathogens by inhibiting the germination of their conidia.

5.6 EXEMPLARY MATING FACTORS FROM FUNGI

The following example lists several mating factors which have been isolated from fungi which are contemplated to be useful in the practice of the present invention.

MFα1-1 mating pheromone from *Saccharomyces cerevisiae* (PID g2144663) (Stoetzler *et al.*, 1976

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYLDLEGDFDVAVLPFSNSTN
NGLLFINTTIASIAAKEEGVSLDKREAEAWHWLQLKPGQPMYKREAEAEAWHWLQLKPGQ
PMYKREADAEAWHWLQLKPGQPMYKREADAEAWHWLQLKPGQPMY (SEQ ID NO:1)

MFα1 mating pheromone from *Saccharomyces cerevisiae*

MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA (SEQ ID NO:12)

MFα2 mating pheromone from *Saccharomyces cerevisiae*

MQPITTASTQATQKDK-SSEKKDNYIIKGLFWDPACVIA (SEQ ID NO:13)

MFm1 mating pheromone from *Schizosaccharomyces pombe*

MDSMANSVSSSSVVNAGNKPAQLNKTVKNYTPKVPYMCVIA (SEQ ID NO:14)

MFm2 mating pheromone from *Schizosaccharomyces pombe* (PID g101045) (Davey, 1992)

MDSIATNTHSSSIVNAYNNNPTDVVKTQNIKNYTPKVPYMCVIA (SEQ ID NO:2)

P-factor mating pheromone from *Schizosaccharomyces pombe* (PID g1076902) (Imai and Yamamoto, 1992)

MKITAVIALLFSLAAASPIPVADPGVVSVSKSYADFLRVYQSWNTFANPDRPNLKKREFE
AAPAKTYADFLRAYQSWNTFVNPDRPNLKKREFEAAPEKSYADFLRAYHSWNTFVNPDRP
NLKKREFEAAPAKTYADFLRAYQSWNTFVNPDRPNLKKRTEEDEENEEEDEEYYRFLQFY
IMTVPENSTITDVNITAKFES (SEQ ID NO:3)

MFBα1 mating pheromone from *Schizophyllum commune* (PID g2133366) (Wendland *et al*, 1995).

MDDFAEFFPTLVLDEPEVARRPARDAEVLAILADAERPGGSNCTAWCVVA (SEQ ID NO:4)

MFBβ1 mating pheromone from *Schizophyllum commune* (PID g1835940) (Vaillancourt and Raper, 1996).

MASSVLARPGPSTVLPAMTRPPPPMAHRAAATPSFARSAQPQ

TDDAVLALLANAEHTEAGEETTARGWCVVA (SEQ ID NO:5)

MFα1 from *Ustilago maydis* (PID g400250) (Bolker *et al.*, 1992)

MLSIFAQTTQTSASEPQQSPTAPQGRDNGSPIGYSSCVVA (SEQ ID NO:7)

MFα2 from *Ustilago maydis* (PID g2133346) (Urban *et al.*, 1996).

MLSIFETVAAAAPVTVAETQQASNNENRGQPGYYCLIA (SEQ ID NO:6)

Mating factor MAT1-1 from *Cochliobolus heterostrophus* (PID g486688) (Turgeon *et al.*, 1993)

MAHARDPTGAEIARFIATRTGAQMVQLMRCIKEPAAQAAFTAKLLVVPPAVSGRPATPEK
ARKALNAFVGFRCYYVTIPMFKSWPMKKLSNLIGLLWEADPNKSLWSLMAKAWSTIRDQI
GKDQAPLDQFFRIICPHLKLPDPASYLEIHGWILTVNEEGDPTISRSADSEFVSIGTGNT
DVALSVEDIITYVQSLGYAHGFILDDNKPSSTFLGQSVSSTLEKNTSAISVTQATPNAAH
ARFLVRNKRRAKRQAVRNASYRASLDQDILIAHQFNPAPVDEHMPDCHSNTAPVLDQCHN
PSPNQFYDGITTLLSDQIPTGQGDAGHLDNAHLFNDYSLPGDVSFITIDDFTTNMPNLID
YDAFRLGADEDVALPIFDDITHI (SEQ ID NO:8)

Mating factor MAT1-2 from *Cochliobolus heterostrophus* (PID g486689) (Turgeon *et al.*, 1993).

MDSTVYSTPPTNSISLAEAIKIAEARFEAAVQGCKDDWHNGNDLVILQDNIPQLFGGILV
EHFKRCVGEVCGFPVELTVMDGGDNYHTLVQMPKNNMRSPQVVSSPQSAQTSPSEQTSIN
LKAVAAGLKKAPRPMNCWIIFRDAMHKHLKAEFPHLTIQEISTRCSHIWHNLSPEAKKPW
QDAAQSAKEEHLRQHPNYKYTPRKPGEKKKRQSRKSKRAAAMTTAPEVLQFQLSPKLIPT
VPEVTDEPPLAANPVTANGNNACPEDVSNCFDPNVFPEIYPEAPMAADFFYNTESIRHSL
LDTEFDIDFNMDTTFALFDDEMLAFRDGADGDATLPSLFEDTY (SEQ ID NO:9)

Vir1 and Vir2 mating pheromone from *Cryphonectria parasitica*

MPSNTQTSNSSMGVNGYSYCVVM (SEQ ID NO:10)

MFα1 mating pheromone from *Cryptococcus neoformans*

MDAFTAIFTTFTSAATSSSEAPRNQEAHPGGMTLCVIA (SEQ ID NO:11)

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. Accordingly, the exclusive rights sought to be patented are as described in the claims below.

WHAT IS CLAIMED IS:

1. A transgenic plant, the genome of which has been augmented through the introduction of a fungal pheromone gene, wherein said gene confers to said transgenic plant resistance to fungal infection, said plant preparable by a process comprising the steps of:

(a) obtaining a polynucleotide composition which comprises one or more fungal pheromone genes;

(b) contacting recipient plant cells with said polynucleotide composition;

(c) regenerating plants from recipient cells which have received said polynucleotide composition; and

(d) identifying a fertile, transgenic plant whose genome has been augmented relative to that of the corresponding nontransgenic recipient cells through the stable introduction of said polynucleotide composition.

2. A transgenic plant, the genetic complement of which has been altered through the addition of a polynucleotide composition comprising a fungal pheromone gene which confers to said plant, resistance to fungal infection.

3. A transgenic plant, the genetic complement of which has been altered through the addition of a polynucleotide composition comprising a pheromone gene which inhibits fungal growth, development, or differentiation in said plant.

4. A transgenic plant, the genetic complement of which has been altered through the addition of a polynucleotide composition comprising a pheromone gene which inhibits fungal conidial sporulation or differentiation in said plant.

5. A transgenic plant, the genetic complement of which has been altered through the addition of a polynucleotide composition comprising a pheromone gene which inhibits appressorium formation in said plant.

6. A transgenic plant having incorporated into its genome a transgene that encodes a fungal pheromone protein or peptide.

7. The transgenic plant according to claim 1, wherein said polynucleotide composition further comprises one or more recombinant vectors.

8. The transgenic plant according to claim 7, wherein said recombinant vectors further comprise one or more genetic elements selected from the group consisting of a promoter, an enhancer, a 5' non-coding region, and a 3' non-coding region.

9. The transgenic plant according to claim 8, wherein said promoter comprises a CaMV 35S, CaMV 19S, *nos*, Adh, sucrose synthase, R-allele or root cell promoter.

10. The transgenic plant according to claim 1, wherein said recipient cells are cotransformed with two or more pheromone genes.

11. The transgenic plant according to claim 10, wherein said pheromone genes are positioned on a single DNA segment, and recipient cells are contacted with said segment.

12. The transgenic plant according to claim 10, wherein said pheromone genes are positioned on two or more DNA segments, and recipient cells are contacted with each of said segments.

13. The transgenic plant according to any preceding claim, wherein said pheromone gene encodes a protein or peptide selected from the group consisting of Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 and MFBβ1.

14. The transgenic plant according to any preceding claim, wherein said pheromone gene comprises a gene isolated from *Magnaporthe*, *Saccharomyces*, *Schizosaccharomyces*, *Cochliobolus*, *Ustilago*, *Schizophyllum*, or *Cryphonectria*.

15. The transgenic plant according to claim 14, wherein said gene is isolated from *Magnaporthe grisea*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Ustilago maydis*, *Cochliobolus heterostrophus*, *Schizophyllum commune* or *Cryphonectria parasitica*.

16. The transgenic plant according to any preceding claim, wherein the coding sequence of said pheromone gene is altered to improve expression of said gene in said plant.

17. The transgenic plant according to claim 16, wherein said gene is altered by random mutagenesis, transposon mutagenesis, site-directed mutagenesis, nucleotide addition or deletion, truncation, or gene fusion.

18. The transgenic plant according to claim 1, wherein said polynucleotide composition further comprises one or more selectable or screenable marker genes.

19. The transgenic plant according to claim 18, wherein said selectable or screenable marker genes are selected from the group consisting of an antibiotic resistance gene, an aequorin gene, a gene encoding a cell wall protein, and a gene encoding an HPRG.

20. The transgenic plant according to any preceding claim, wherein pheromone gene is selected from the group consisting of *Vir1*, *Vir2*, *MFα1-1*, *MFα1*, *MFα2*, *MFα*, *MFm1*, *MFm2*, *MAT1-1*, *MAT1-2*, *MFBα1* and *MFBβ1*.

21. The transgenic plant according to any preceding claim, wherein said transgenic plant is selected from the group consisting of corn, wheat, cotton,

flax, barley, rye, oats, rice, soybean, tobacco, fruit, berry, nut, ornamental, pasture grass, turf grass and shrub.

22. A fertile, transgenic plant, the genome of which has been augmented by the introduction of a polynucleotide composition comprising an exogenous gene encoding a fungal pheromone, wherein said gene is positioned under the control of an inducible or tissue-specific promoter or enhancer, provided said transgenic plant exhibits resistance to fungal infection, and wherein said gene is transmittable through normal sexual reproduction of the transgenic plants to subsequent generation plants.

23. A fertile, transgenic plant, the genome of which has been augmented by the introduction of a polynucleotide composition comprising a gene encoding a mating-type specific pheromone gene, wherein said transgenic plant exhibits fungal resistance, and wherein said gene is transmittable through normal sexual reproduction of the transgenic plant to subsequent generation plants.

24. Progeny of the plant according to any preceding, wherein said progeny comprise said pheromone gene.

25. Seeds obtained from a plant or progeny according to any preceding claim, wherein said seeds comprise said pheromone gene.

26. Cells obtained from a plant, progeny, or seed according to any preceding claim, wherein said cells comprise said pheromone gene.

27. A process for producing a fertile transgenic plant resistant to fungal infection, comprising the steps of (i) establishing a regenerable culture from a plant to be transformed, (ii) transforming said culture with a polynucleotide composition comprising a fungal pheromone gene, (iii) identifying or selecting a transformed cell line and (iv) regenerating a fertile transgenic plant therefrom, wherein said fungal pheromone gene is transmitted through a complete sexual cycle of said transgenic plant to its progeny, wherein said progeny comprises said fungal pheromone gene, and wherein said fungal pheromone gene is chromosomally integrated.

28. The process according to claim 27, wherein said polynucleotide composition further comprises one or more recombinant vectors.

29. The process according to claim 28, wherein said recombinant vectors further comprise one or more genetic elements selected from the group consisting of a promoter, an enhancer, a 5' non-coding region, and a 3' non-coding region.

30. The process according to claim 29, wherein said promoter comprises a CaMV 35S, CaMV 19S, *nos*, Adh, sucrose synthase, R-allele or root cell promoter.

31. The process according to claim 27, wherein said culture is cotransformed with two or more pheromone genes.

32. The process according to claim 31, wherein said pheromone genes are positioned on a single DNA segment, and said culture is transformed with said segment.

33. The process according to claim 31, wherein said pheromone genes are positioned on two or more DNA segments, and said culture is transformed with each of said segments.

34. The process according claim 27, wherein said pheromone gene encodes a protein or peptide selected from the group consisting of Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 and MFBβ1.

35. The process according to any one of claims 27 through 34, wherein said pheromone gene comprises a gene isolated from *Magnaporthe*, *Saccharomyces*, *Schizosaccharomyces*, *Cochliobolus*, *Ustilago*, *Schizophyllum*, or *Cryphonectria*.

36. The process according to claim 35, wherein said gene is isolated from *Magnaporthe grisea*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Ustilago maydis*, *Cochliobolus heterostrophus*, *Schizophyllum commune* or *Cryphonectria parasitica*.

37. The process according to any one of claims 27 through 36, wherein the coding sequence of said pheromone gene is altered to improve expression of said gene in said plant.

38. The process according to claim 37, wherein said gene is altered by random mutagenesis, transposon mutagenesis, site-directed mutagenesis, nucleotide addition or deletion, truncation, or gene fusion.

39. The process according to claim 27, wherein said polynucleotide composition further comprises one or more selectable or screenable marker genes.

40. The process according to claim 39, wherein said selectable or screenable marker genes are selected from the group consisting of an antibiotic resistance gene, an aequorin gene, a gene encoding a cell wall protein, and a gene encoding an HPRG.

41. The process according to any one of claims 27 through 40, wherein said pheromone gene is selected from the group consisting of *Vir1*, *Vir2*, *MFα1-1*, *MFα1*, *MFα2*, *MFα*, *MFm1*, *MFm2*, *MAT1-1*, *MAT1-2*, *MFBα1* and *MFBβ1*.

42. The process according to any one of claims 27 through 41, wherein said transgenic plant is selected from the group consisting of corn, wheat, cotton, flax, barley, rye, oats, rice, soybean, tobacco, fruit, berry, nut, ornamental, pasture grass, turf grass and shrub.

43. The process of any one of claims 27 through 42, further comprising obtaining progeny of said fertile, transgenic plant, wherein said progeny is a fertile, transgenic plant that comprises a gene encoding a pheromone selected from the group consisting of Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 and MFBβ1.

44. The method of claim 43, further comprising breeding said progeny with a non-transgenic plant, to prepare a fertile, transgenic plant that comprises a gene encoding a pheromone selected from the group consisting of Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 and MFBβ1.

45. The process of any one of claims 27 through 70, further comprising preparing a seed from one or more fertile, transgenic plants that comprises the gene encoding fungal resistance, wherein said seed comprises one or more genes encoding Vir1, Vir2, MFα1-1, MFα1, MFα2, MFα, P-factor, MFm1, MFm2, MAT1-1, MAT1-2, MFBα1 or MFBβ1.

46. The process of claim 45, further comprising cultivating said seed to prepare a fertile, transgenic plant that comprises a gene encoding a fungal mating-type specific pheromone.

47. A method of using a DNA segment that encodes a fungal pheromone protein or peptide, comprising the steps of:

a) preparing a recombinant vector in which a fungal pheromone protein or peptide-encoding DNA segment is positioned under the control of a promoter;

b) introducing said recombinant vector into a host cell;

c) culturing said host cell under conditions effective to allow expression of the encoded fungal pheromone protein or peptide; and

d) collecting said expressed pheromone protein or peptide.

48. A method for detecting a nucleic acid sequence encoding a fungal pheromone protein, comprising the steps of:

a) obtaining sample nucleic acids suspected of encoding a fungal pheromone protein;

b) contacting said sample nucleic acids with an isolated nucleic acid segment encoding said pheromone protein under conditions effective to allow hybridization of substantially complementary nucleic acids; and

c) detecting the hybridized complementary nucleic acids thus formed.

49. A fungicidal composition comprising a mating type specific fungal pheromone.

50. A method of inhibiting fungal infection in a plant comprising applying to said plant a fungicidally-effective amount of a mating type specific fungal pheromone.

51. A method of inhibiting maturation of fungal conidia comprising contacting said conidia with an amount of a mating type specific fungal pheromone effective to inhibit said maturation.

52. A method of inhibiting growth of a fungus comprising contacting said fungus with an amount of a mating type specific fungal pheromone effective to inhibit said growth.

53. A method of preventing fungal infection in a plant comprising contacting said plant with an amount of a mating type specific fungal pheromone effective to prevent fungal infection in said plant.

54. A method of preventing fungal colonization of a plant comprising contacting said plant with an amount of a fungal mating pheromone effective to prevent fungal colonization of said plant.

55. A method of treating fungal infection of a host, such as a plant or an animal, comprising contacting said host with a fungicidally-effective amount of a mating type specific fungal pheromone.

```
Cp-Vir1 & 2   mpsntqts----------------------------Nssmgvngysyc VVM
Cn-MFalpha:   mdaftaiftftsaatsseapr---------N-qeahpggmt1-CVIA
Sc-MFa1:      mqpsta--t-aapkektssekkd---------N-yiikgvfwdpaCVIA
Sc-MFa2:      mqpittastqatqkdk-ssekkd---------N-yiikglfwdpaCVIA
Sp-MFm1:      mdsmansvssssvvnagnkpaqlnktvk---N-ytpkvpym---CVIA
SP-mFm2:      mdsiatnthsssivnaynnnptdvvktgnikN-ytpkvpym---CVIA
Um-MFa1:      mlsifaq-ttqtsasepgqptapggr-----N-dngspigyss-CVVA
Um-MFa2:      mlsifetwaaaapvtvaetqqas---------N-nenrgqpgyy-CLIA
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FIG. 1

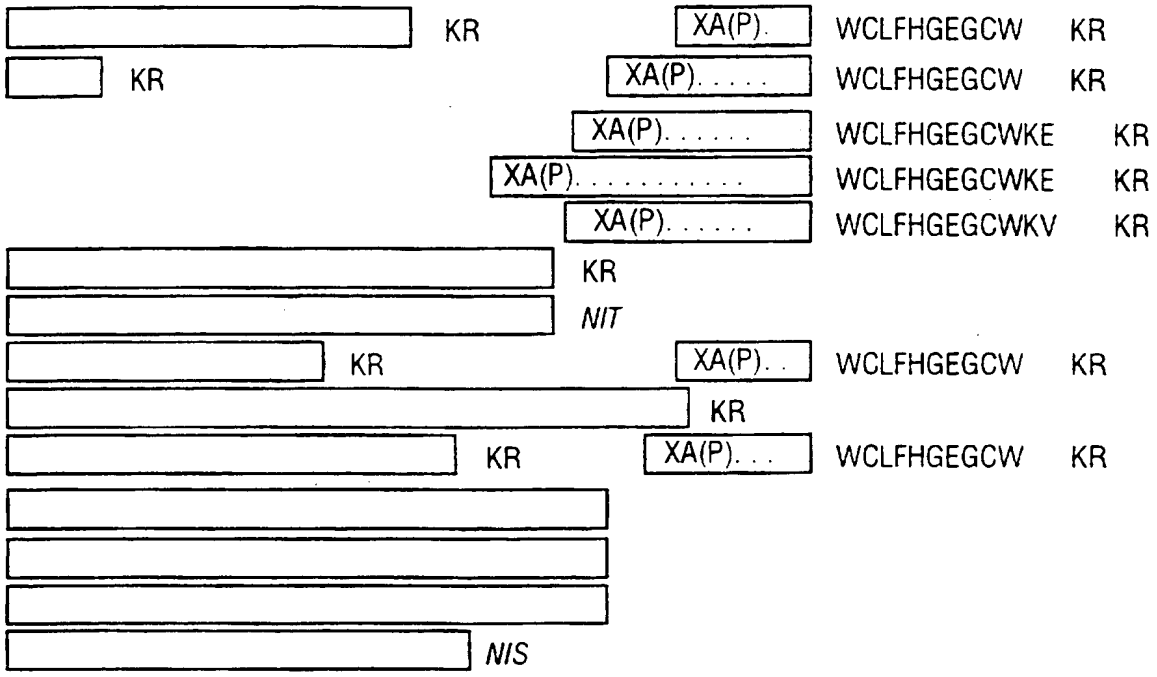

FIG. 2A

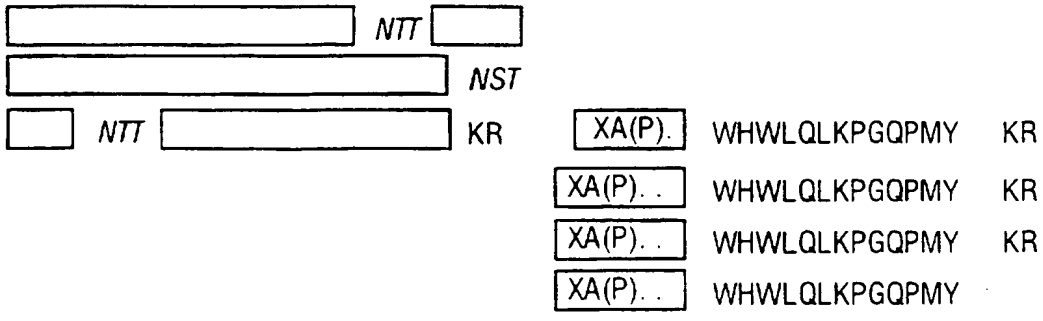

FIG. 2B

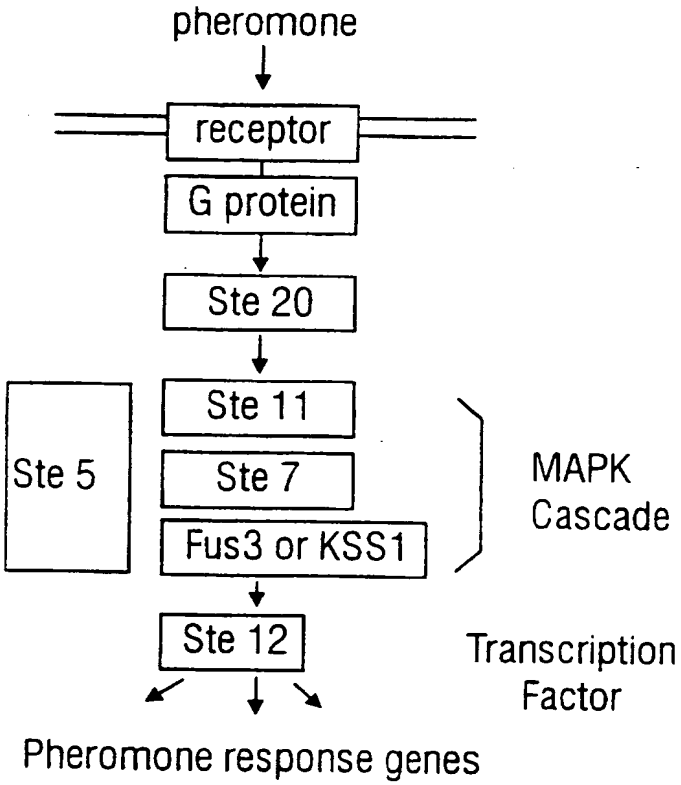
pheromone
receptor
G protein
Ste 20
Ste 11
Ste 5
Ste 7
Fus3 or KSS1
MAPK
Cascade
Ste 12
Transcription
Factor
Pheromone response genes

FIG. 3

FIG. 4A

FIG. 4B

FIG. 4C

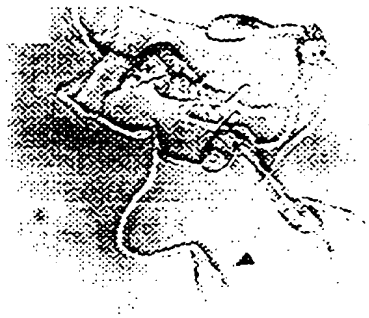

FIG. 4D

FIG. 4E

FIG. 4F

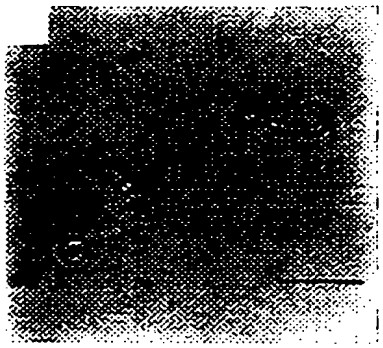

FIG. 4G

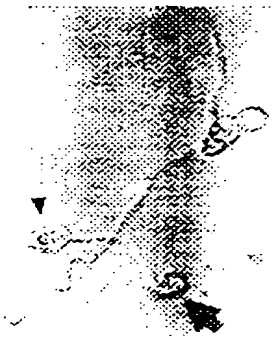

FIG. 4H

WATER CONTROL

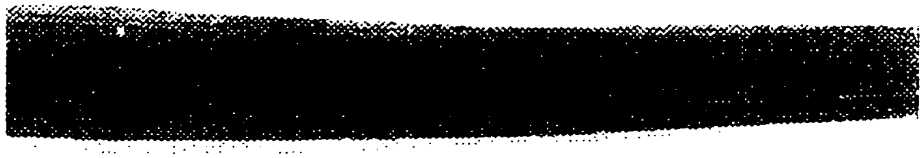

FIG.5A

CONIDIA

FIG.5B

α – FACTOR

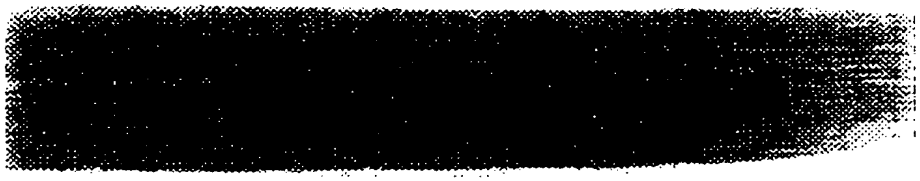

FIG.5C

CONTROL PEPTIDE

FIG.5D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10364

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C07K 14/37; C12N 5/04; C12P 21/00

US CL :435/69.1; 530/371; 536/24.32; 800/205, 250

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.4; 530/300, 324, 325, 350, 371; 536/24.3, 24.32; 800/205, 250

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN:Medline Biosis Embase CAPlus WPIDS JAPIO PATOSEP PATOSWO

Search terms: pheromone, fungus, transgenic, plant, vir?, mfalpha?, p-factor, mfm?, mat1?, mfb?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMAI Y. et al. The fission yeast mating pheromone P-factor: its molecular structure, gene structure, and ability to induce gene expression and G1 arrest in the mating partner. Genes Dev. February 1994. Vol. 8. No. 3. pages 328-338, see entire document.	47-49
X	KANG S. et al. Isolation of the mating-type genes of the phytopathogenic fungus Magnaporthe grisea using genomic subtraction. Genetics. October 1994. Vol. 138. No. 2. pages 289-296, see entire document.	47, 48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US97/10364

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DAVEY J. Mating pheromones of the fission yeast Schizosaccharomyces pombe: purification and structural characterization of M-factor and isolation and analysis of two genes encoding the pheromone. EMBO J. March 1992. Vol. 11. No. 3. pages 951-960, see entire document.	49
X,P	BECKERMAN J.L. et al. Inhibition of pathogenicity of the rice blast fungus by Saccharomyces cerevisiae alpha-factor. Science. May 1997. Vol. 276. No. 5315. pages 1116-1119, see entire document.	49-55